Targeting mammalian target of rapamycin (mTOR) signaling in the Han:SPRD rat, a model for polycystic kidney disease

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Summary

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the progressive development of innumerable cysts in both kidneys, which distort the normal kidney architecture, leading to a loss of renal function necessitating renal replacement therapy and/or kidney transplantation. The pathogenesis of ADPKD is complex, but a hallmark of the disease is enhanced renal tubular cell proliferation, which can be revealed in the epithelium that lines the cysts. The protein kinase mTOR is a central controller of cell proliferation and growth, and therefore we hypothesized that the mTOR signaling pathway could be dys-regulated in polycystic kidney disease (PKD) and treatment with mTOR inhibitors could retard disease progression in PKD. In a previous study, we showed that oral administration of rapamycin, an mTOR inhibitor, inhibited cyst growth, preserved renal functions and suppressed mTOR activities in the Han:SPRD rat, a rat model for PKD.

To determine if this was a class effect of the mTOR inhibitors, we examined the effect of everolimus, an analogue of rapamycin, on disease progression in the Han:SPRD rat. We showed that a 5 week everolimus treatment by gavage feeding prevented the deterioration of renal function and cyst growth associated with decreased cell proliferation in cystic kidneys.

Two mTOR inhibitors are currently being tested clinically in PKD patients. Adverse effects of mTOR inhibitors have been reported clinically that may constrain their potential use in PKD. Pulse treatment may increase treatment tolerability. Thus, we examined the effect of pulse and continuous everolimus treatment in Han:SPRD rats. Four-week-old heterozygous polycystic and wild-type rats were administered everolimus or vehicle by gavage feeding for 5 weeks, followed by 7 weeks without treatment, or continuously for 12 weeks. Cessation of everolimus did not allow renal cyst re-growth up to 7 week post-withdrawal. The partial re-activated S6 kinase and

recovery of cell proliferation after everolimus withdrawal may contribute to the striking non-cystic renal parenchyma enlargement and glomerular hypertrophy. Both treatment regiments ameliorated kidney function, preserved glomerular-tubular connection and reduced proteinuria. Pulse treatment in the early age delayed cyst development but led to striking glomerular and parenchymal hypertrophy which might have a major impact when assessing the effect of mTOR inhibitor treatment in patients with polycystic kidney disease.

To explore the effects and mechanism of a wide range of everolimus levels (0.01 nM to 100 nM) on tubular epithelial cells, we cultured primary tubular epithelial cells from wild type (+/+) and Cy/+ Han:SPRD rats in 2D and 3D system. Cy/+ cells proliferated faster than +/+ cells as shown by a much lower population doubling time in Cy/+ cells. 0.1 nM everolimus reduced the cell number and DNA synthesis with no effect on apoptosis. Phosphorylation of mTOR, S6 kinase and S6 in Cy/+ were dose-dependently reduced by everolimus starting from 0.1 nM in parallel with effects on cell number and DNA synthesis. The aggregate diameter of Cy/+ was higher compared to +/+ and everolimus selectively reduced the Cy/+ aggregate size. These data showed that an everolimus level of 0.1 nM affects cell proliferation, aggregate size and phosphorylation of mTOR pathway effectors.

Taken together we revealed the critical role of mTOR in renal tubular epithelial cell proliferation and cyst growth in the Han:SPRD rat. Our results have substantially extended the knowledge of the role of mTOR inhibitors in polycystic kidney disease and our data may have potential clinical impacts when considering a longterm therapy using everolimus in ADPKD.

Zusammenfassung

Die autosomal dominante polyzystische Nierenerkrankung (ADPKD) ist charakterisiert durch die fortschreitende Ausbildung von unzähligen Zysten in beiden Nieren, welche die normale Nierenarchitektur zerstört und schliesslich zu einem Verlust der Nierenfunktion und der Notwendigkeit für eine Nierenersatztherapie und Nierentransplantation führt. Die Pathogenese der ADPKD komplex ist; ein Hauptmerkmal ist die verstärkte Proliferation von Tubulusepithelzellen, welche die Zysten auskleiden. Die Proteinkinase mammalian target of rapamycin (mTOR) ist eine zentrale Steuerung der Zellproliferation und des Wachstums. Wir stellten deshalb die Hypothese auf, dass der mTOR Signalweg in polyzystischen Nieren dysreguliert ist und dass die pharmokologische Hemmung des Signalweges die Krankheitsprogression vermindert. In einer früheren Studie konnten wir zeigen, dass die orale Gabe von Rapamycin, ein mTOR Inhibitor, das Zystenwachstum hemmt, die Nierenfunktion normalisiert und die mTOR Aktivitäten unterdrückte in einem Tiermodell für die polyzystische Nierenerkrankung (Han:SPRD Ratte).

Um festzustellen, ob unser Ergebnis Rapamycin spezifisch ist oder um es sich um eine Wirkung der Substanzklasse der mTOR Inhibitoren handeltet, untersuchten wir die Wirkung von Everolimus, dem Analogon von Rapamycin, auf das Fortschreiten der Erkrankung in der Han:SPRD Ratte. In diesem Versuch konnten wir zeigen, dass die Behandlung mit oral appliziertem Everolimus für 5 Wochen die Nierenfunktion verbessert und damit verbunden, das Zystenwachstum und die Zellproliferation reduziert wird.

Zwei mTOR Inhibitoren werden derzeit klinisch in ADPKD Patienten erprobt, allerdings könnten mTOR Inhibitor assozierte Nebenwirkungen eine klinische Anwendung in ADPKD einschränken. Pulse Behandlung kann die Verträglichkeit der

Behandlung erhöhen. Wir untersuchten deshalb den Effekt von Puls und kontinuierlicher Everolimus Behandlung in Han:SPRD Ratten. In vier Wochen alten heterozygote polyzystische und Wildtyp-Ratten wurden Everolimus oder das Vehikel während 5 Wochen über eine Magensonde appliziert, gefolgt von 7 Wochen ohne Behandlung oder kontinuierlich über 12 Wochen. Die Puls Everolimus Behandlung verhinderte ein Zystenwachstum auch 7 Wochen nach Beendigung der Therapie trotz einer Teilaktivierung der S6 Kinase und Zunahme der Zellproliferation. Auffallend waren eine nicht-zystische Zunahme des Nierenparenchyms und die Vergrösserung des glomerulären Volumens. Puls wie auch die kontinuierliche Therapie hatten die Nierenfunktion verbessert, die glomeruläre-tubuläre Verbindung erhalten und die Proteinurie vermindert. Eine Puls Behandlung zu Beginn der Erkrankung verzögert die Zystenbildung, aber führt zu einer ausgeprägten parenchymalen und glomerulären Hypertrophie, was die Beurteilung der Wirkung von mTOR Inhibitor bei Patienten mit polyzystischer Nierenerkrankung beeinflussen könnte.

Um die Auswirkungen und Mechanismen einer Everolimuskonzentration von 0,01 nM bis 100 nM auf tubuläre Epithelzellen zu untersuchen, kultivierten wir primäre tubuläre Epithelzellen aus dem Wildtyp (+/+) und Cy/+ Han:SPRD Ratten in 2D- und 3D-Kultursystemen. Cy/+ Tubulusepithelzellen vermehrten sich im Vergleich zu +/+ Zellen schneller, dargestellt durch die wesentlich geringere Populationverdopplungszeit. 0.1 nM Everolimus reduzierte die Zellanzahl und die DNA-Synthese ohne eine Apoptose zu induzieren. Die Phosphorylierung von mTOR, S6 und S6 Kinase in Cy/+ wurde dosisabhängig ab einer Everolimuskonzentration von 0.1 nM reduziert und parallel dazu die Zellanzahl und die DNA-Synthese vermindert. Der Durchmesser von Cy/+ Zellaggregaten war im Vergleich zu +/+ Aggregaten grösser und Everolimus reduzierte selektiv den Durchmesser von

solchen Cy/+ Zellaggregate. Diese Daten zeigten, dass eine Everolimuskonzentration von 0.1 nM die Zellproliferation, die Aggregatgrösse und die Phosphorylierung von Effektoren des mTOR Signalwegs beeinflusst.

Zusammenfassend haben wir die entscheidende Rolle des mTOR Signalweges für die tubuläre epitheliale Proliferation und Zystenwachstum in der Han:SPRD Ratte untersucht. Unsere Ergebnisse haben das Wissen über die Wirkmechanismen von mTOR Inhibitoren bei der polyzystischen Nierenerkrankung wesentlich erweitert und unsere Daten haben einen Einfluss auf eine mögliche langfristige Anwendung von mTOR Inhibitoren in ADPKD Patienten.

Contents

Chapter 1

Introduction

1 Introduction

Cystic kidney diseases are a common cause of end stage renal failure. Most forms of cystic kidney diseases are hereditary, but renal cysts can also be acquired by aging and treatments like dialysis. Onset of cystic kidney disease varies from newborn to seven or eight decades. Besides kidney, liver, pancreas and cardiovascular system are the most commonly affected. Cystic kidney diseases are monogenic diseases and more than 20 genes have been linked to different forms of cystic kidney diseases in the last 15 years. Diagnosis can be done by imaging, e.g. ultrasound, CT and MRI. Recently, gene sequencing has been used as molecular diagnosis for these diseases. Some common features of these diseases are aberrant proliferation, apoptosis, loss of cellular polarity, and secretion. Recently, focus has moved to the study of primary cilium, where most mutated genes for cystic kidney diseases are located. Two-hit and third-hit hypothesis were proposed to further understand the mechanisms. Advances in research on these diseases in recent years have attributed to the availability of various new animal and cell culture models. At present, there is no treatment for cystic kidney diseases. However, molecular studies have prompted designing of clinical trials targeting several key molecular pathways in recent years. In this study, we focused on mTOR, the central controller of cell proliferation and growth. We studied the effect of mTOR inhibition in Han:SPRD rat model of autosomal dominant polycystic kidney disease (ADPKD), the most common form of cystic kidney disease.

1.1 Classification of renal cystic diseases

Cystic kidney diseases can be categorized into primary and secondary hereditary forms, depending on whether renal cyst is the main manifestation. Other cystic kidney diseases can be acquired because of non-genetic reasons like differentiation abnormalities and dialysis treatment.

1.1.1 Autosomal dominant polycystic kidney disease (ADPKD)

Autosomal dominant polycystic kidney disease (ADPKD) accounts for 5-10% of patients with end stage renal disease who require renal replacement therapy (dialysis or renal transplantation) [1-3]. The incidence of ADPKD is between 1:400 to 1:1000 and it affects 12.5 million people worldwide [1, 4]. Although 50% ADPKD patients progress to end stage renal failure, the onset of clinical symptoms and renal function decline vary from first to eighth decades of life with an average age of onset in the fifth decade of life [2]. Renal cysts can even be diagnosed in utero or at birth and 2-5% of ADPKD patients present severe neonatal morbidity and mortality [5, 6]. ADPKD is characterized by continuous growth of kidney (up to more than 10 fold) and renal cysts with an average annual kidney growth rate of more than 5% [4, 6, 7]. Renal function remains stable when kidney volume increases from normal size (150-200 cm^3) in adolescence to 1,500 cm^3 in the fourth or fifth decades of adulthood by when 65% of normal renal parenchyma has been replaced by non-functional nephrons owing to cyst expansion and fibrosis [2, 8]. The dissociation between steady cyst growth and the late decline of renal function can be explained by compensatory mechanisms (glomerular hyperfiltration) that maintain the normal renal function for decades. Cysts in Liver (80% patients), pancreas (10% patients), seminal vesicles, spleen and arachnoid membrane are often observed in ADPKD [8]. Other extrarenal

manifestations include intracranial aneurysms, aortic aneurysms and cardiac valve defects. The common clinical manifestation of ADPKD include hypertension, hematuria, polyuria, flank pain, urinary tract infections, uric acid stones and renal stones (20% patients) [8].

Macroscopically, enormous renal cysts exist bilaterally and derive from all segments of nephron, although only 1-5% nephrons are affected [1, 9]. The size of renal cyst varies up to several centimetres in diameter and throughout cortex and medulla region. Microscopically, the shape of cyst-lining epithelial cells range from columnar to cuboidal or flattened, implying the origin of renal cyst from different nephron segments. Cyst-lining epithelia are often surrounded by a layer of thickened basement membrane and a growing vasculature network [2, 7]. Inflammatory infiltration and interstitial fibrosis caused by increased fibroblasts and collagen are observed in ADPKD [2, 10].

1.1.2 Autosomal recessive polycystic kidney disease (ARPKD)

The prevalence of autosomal recessive polycystic kidney disease (ARPKD) is approximately 1:20,000 live births [2, 4, 5]. It often causes fetal and neonatal death (30% cases) mainly because of lung hypoplasia due to oligohydramnios. Amongst the survivors, 20-45% patients progress to end stage renal failure and need renal replacement therapy by age 15. Extrarenal manifestations include portal hypertension and biliary dysgenesis resulting in hepatic fibrosis. ARPKD patients in adolescence and adulthood present less severe renal disease, but more severe liver complications.

Macroscopically, ARPKD patients develop bilateral renal cysts resulting kidney enlargement, but their kidneys retain reniform unlike ADPKD kidneys. The kidney

appears spongy, and there is no clear separation of cortex and medulla [5]. ARPKD renal cysts mainly originate from collecting ducts presenting fusiform, ecstatic dilated shape and radiated from medulla to the cortex. Microscopically, a single layer of cuboidal epithelium lines the cysts, surrounded by abnormal deposition of extracellular matrix [2, 5].10-90% of collecting ducts are affected and this is associated with differential severity of renal disease. Unlike ADPKD, cystic lesions in ARPKD retain the structure of afferent and efferent connection.

1.1.3 Other primary genetic renal cystic diseases

Other primary genetic renal cystic diseases include nephronophthisis (NPHP), medullary cystic kidney disease (MCKD) and glomerulocystic kidney disease [2]. NPHP cause renal failure in children or young adults. Macroscopically, NPHP kidneys are small and shrunken. Numerous small cysts exist in corticomedullary junction and they originate from distal convoluted and collecting tubules.

Typical onset of end stage renal failure of MCKD type 1 is in sixth decades of life. Macroscopically, MCKD kidneys appear normal or slightly small. Numerous small renal cysts locate in corticomedullary junction. Average onset of end stage renal failure of MCKD type 2 is 32 years of age. Renal cysts are detected in inner medulla and probably inner medullar collecting tubule origin.

The onset of glomerulocystic kidney disease can be early when associated with developmental dysplasia or later when associated with ADPKD. Glomeruli with dilated Bowman`s space are often detected. Glomerular tufts are often reduced or collapsed.

1.1.4 Secondary hereditary renal cystic disease

Secondary hereditary renal cystic diseases include tuberous Sclerosis Complex (TSC), von Hippel-Lindau Disease (VHL), Bardet-Biedl Syndrome (BBS), and Oral-Facial-Digital Syndrome Type 1 (OFD-1) [2, 4]. Study of secondary hereditary renal cystic diseases provides important insight into mechanism of cystogenesis.

TSC is inherited as an autosomal dominant trait. Renal cysts are rarely seen but presented as ADPKD phenotype, probably because genes responsible for these two diseases are immediately adjacent in the same chromosome.

VHL is multisystem neoplastic disorder and also inherited in an autosomal dominant trait. Renal cysts are seen in 70% VHL patients. VHL is characterized by high vascularisation due to abnormal angiogenesis.

BBS and OFD-1 are two very rare diseases, with the the incidence 1:160,000 and 1:50,000 live birth respectively.

1.1.5 Nonhereditary renal cystic disorders

Nonhereditary renal cystic disorders include Simple Renal Cysts, Acquired Cystic Kidney Disease, Multicystic Renal Disease and Medullary Sponge Kidney [2]. These cystic diseases are caused by aging, hemodialysis, and developmental abanormalities.

1.2 Genetics of polycystic kidney disease

Polycystic kidney diseases (PKD) are monogenic disorders and many genes and encoded proteins are identified and characterized.

1.2.1 ADPKD genes and proteins

ADPKD is caused by mutation in *PKD1* or *PKD2* genes. *PKD1*, localized on chromosome 16p13.3, accounts for 85% cases and is related to severe disease phenotype [4]. *PKD2*, localized on chromosome 4q21 and accounts for the rest 15% cases related to less severe disease phenotype. The average onset of ESRD in *PKD1* is 54.3 years, which is 10-20 years younger than in *PKD2*. Thus, fewer patients with *PKD2* mutation reach ESRD during their lifetime. The disease severity is depends on the number of cysts formed at an early age, not because of the growth rate of cysts [11].

PKD1 gene, with 46 exons and 50 kb in length, encodes a transcript with open reading frame (ORF) of 12,909 bp. *PKD2* gene, with 15 exons and 68 kb in length, has an ORF of 2904 bp. A large number of mutations were identified in *PKD1* and *PKD2* genes. The mutation type (either truncating or missense mutation) is not strongly associated with disease phenotypes, however the mutation location is associated with the severity of diseases [4]. Patients with mutation in 5` region of *PKD1* developed more severe disease than in 3` region of *PKD1* [6].

PKD1 and *PKD2* encode polycystin 1 (PC1 or TRPP1) and polycystin 2 (PC2 or TRPP2) respectively, which belong to the subfamily (TRPP) of transient receptor potential (TRP) channels. The expression of PC1 is largely reduced in adult as compared to fetal kidney, indicating a role transition of polycytin from tubular construction during development period to maturation maintenance in adult life [12, 13]. In mouse kidney, PC1 expression peaks at embryonic (E) day 15; it falls to a low level two weeks after birth and this level is maintained in adult life [9]. PC1 is a large trans-membrane protein (with 4303 amino acids and around 460 kDa) with 11 transmembrane domains (1032 amino acids), a long extracellular domain (3074 amino acids) and a short intracellular C-terminal region (197 amino acids). Overall

PC1 has a structure of a receptor or an adhesion molecule. It is a distant TRP homolog and may have non-selective cation channel activity by its final 6 transmembrane domains. Although PC1 is cleaved in the extracellular portion at the G protein-coupled receptor proteolytic site (GPS) domain and results in a C-terminal fragment (150 kDa) and N-terminal fragment, they remain tethered and the cleavage is important for PC1 function. C-terminal tail of PC1 contains multiple sites to interact with proteins to regulate cell proliferation and growth. Fluid flow inhibits the cleavage of C-terminal tail of PC1, which may migrate to nucleus and regulate gene transcriptions.

PC1 can form a complex with E-cadherin and a,β, and γ-catenins in adherens juctions to regulate cell-cell interaction. In ADPKD PC1/E-cadherin complex is disrupted.

PC2 (968 amino acids and around 110 kDa) is a non-selective cation channel with 6 transmembrane domains and contain a short N-terminal cytoplasmic region with a ciliary targeting motif. The cytoplasmic C-terminal of PC2 may contain a calcium-binding motif (EF-hand) and coiled-coil domain, which is important for interaction with PC2 and other TRP family members (PC1, TRPC1 and TRPV4). PC2 can directly modulate cell cycle by interacting and sequestering transcription factor Id2 [9].

PC1 is proposed to function as a G protein-coupled receptor (GPCR) when expressed alone [9]. Comexpression of PC2 with PC1 strongly suppresses the activation of heterotrimeric G proteins by PC1 and calcium channel activity of PC2 [9, 14]. PC1/2 complex functions as a mechanosenser to sense the fluid flow. Urine flow shear stress may activate PC1/2 complex and subsequent lead to conformational change of PC1, resulting in activation of PC1 mediated G protein signaling and PC2-mediated calcium entry.

Polycystins are localized in various subcellular organelles including primary cilium, endoplasmic reticulum, centrosome, mitotic spindles and also involved in cell-cell and cell-matrix interactions. Subcellular transport and localization of polycystins can be regulated by chemical chaperones, proteasome inhibitors, phosphorylation and multiple interaction with adaptor proteins [6, 9, 15-17]. It has been shown that polycystins can be packaged into exosomes and preferentially transported to primary cilia of kidney and biliary epithelial cells.

1.2.2 ARPKD genes and proteins

Mutation in *PKHD1* (polycystic kidney and hepatic disease 1) gene causes ARPKD. *PKHD1* (6q21) has 67 exons and is 470 kb in length and is maybe the largest identified disease gene up to date. It has many spliced products and with the longest ORF of 12,222 bp. The high level of allelic heterogeneity in *PKHD1* gene is caused by truncating mutation (40%) or missense mutation (60%). Two truncating mutations always associated with the most severe cases, indicating a complete penetration of the mutated alleles, which produce less functional protein products. Animal studies have shown that *PKHD1* gene is limited expressed in kidney, liver, pancreas and lung, whereas most PKD genes are widely expressed. The tissue specific expression pattern of *PKHD1* gene is regulated by the transcription factor hepatocyte nuclear factor-1 β (HNF-1β), which bind the proximal promoter of *PKHD1* gene [5].

Fibrocystin (4074 amino acids and 447 kDa), the protein product of *PKHD1* gene, has a large extracellular N-terminal portion (3860 amino acid), a single transmembrane domain and a short intracellular C-terminal tail (192 amino acids) [6]. The proprotein convertase site in the extracellular portion of fibrocystin is thought to

be cleaved. Like PC1 the C-terminal tail of fibrocystin can be cleaved and migrate to nucleus.

Fibrocystin is expressed in plasma membrane, centrosomes, basal bodies, primary cilia and may also involve in cell-cell interactions in epithelia [5]. Like PC1, fibrocystin can also form complex with PC2 and packaged in exosome for intracellular transporting. The function of fibrocystin is largely unknown, but the structure and localization of fibrocystin suggests a function as receptor protein involved in the regulation of cell adhension, repulsion, proliferation and differentiation.

1.2.3 Genes and proteins for other cystic kidney diseases

More than 20 genes and proteins responsible for other cystic kidney diseases have been identified and characterized. Study of these disorders provided great insight of the mechanism of cyst formation and development. Like PC1/2 and fibrocystin, these proteins mostly located in primary cilia, centrosome, and basal body and expressed in basal-lateral membrane for cell-cell and cell-matrix interaction. The most important finding in the field of PKD research is the role of primary cilium in cystogenesis since mutation of these genes leads to defect of primary cilium.

1.3 Mechanisms of cyst genesis and development

PKD is characterized by the expansion of fluid filled renal cysts. Initial studies on abnormal proliferation and secretion of cyst lining epithelia led to further elucidation of fundamental defects in planar cell polarity, epithelial polarity, proliferation, apoptosis, cell-cell interaction and cell-matrix interaction [2, 18].

1.3.1 Planar cell polarity

Planar cell polarity (PCP) means the spatial organization of cells along a tissue plan that is perpendicular to the apical-basal axis [18]. PCP proteins were initially found in *Drosophila* and consist of two pathways. The core PCP pathway involves the membrane proteins Frizzled, Strabismus/Van Gogh, and Flamingo/Starry Night and the cytoplasmic proteins Dishevelled, Prickle, and Diego. The second PCP pathway involves two protocadherins, Fat and Dachsous, and transmembrane protein called Four-jointed, and the transcriptional repressor Atrophin.

PCP plays an important role in embryonic development by regulating cell migration, cell orientation, and mitotic spindle orientation, and other morphogenetic processes. It has been shown for the first time by Fischer *et al* that abnormal PCP is involved in PKD. The study demonstrated that disoriented cell division led to tubular dilation/ cyst formation and defective PCP presented in the early stage of cystogenesis. Furthermore, a recent study showed that mutation of the PCP protein Fat4 in mice led to the disruption of orientation of cell division and subsequently induced polycystic kidney disease. Moreover, secreted Frizzled-related protein 4 (sFRP4) is found to be up-regulated in human ADPKD and several different animal models of PKD [6]. In addition, sFRP4 selectively influenced several members of Wnt signaling and promoted cystogenesis in zebrafish embryos. Therefore PCP disruption could be the key step for transformation of a renal epithelial tubule into a cyst.

The aberrant PCP in PKD may be caused by defective cilium. Inactivation of ciliogenic gene *Kif3a* or *Ift20* disrupted cilia formation and led to randomized orientation of cell division. Cilium regulate PCP may through the β-catenin independent non-canonical Wnt signaling pathway. Simons et al. showed that the protein inversin functions as a switch between canonical and non-canonical Wnt

signaling pathways and that fluid flow inhibits canonical Wnt signaling and thereby facilitate normal tubule development [19]. Loss of cilium by inactivating PKD genes switches on β-catenin dependent canonical pathway but inhibits PCP/ non-canonical signaling. Cilium regulates PCP may also via Fat/Dachsous pathway. *Fat4* is found to be localized in primary cilium and homozygous deletion of *Fat4* leads to tubule dilation in mouse kidney [9, 20].

1.3.2 Membrane polarity of epithelial cells

Cell membrane polarity is the characteristic of cellular asymmetry at morphologic and functional level. Acquisition of membrane polarity is the fundamental hallmark of renal tubular development and maturation [2]. Membrane polarity can be different or opposite for some proteins during development and after maturation. PC1 during fetal phase is primarily located at the basal membrane but down-regulated and restricted to apical-lateral membrane after maturation in renal collecting ducts.

Loss of membrane polarity is observed in PKD. Some proteins are mispolarized into apical membrane. These include EGFR/Her2/neu (ErbB2) complex, Nak-ATPase (a1/β2 subunits), calpactin, ankyrin and fodrin. Some proteins such as NaK2Cl (BSC2) symporter and cathepsin L are mispolarized into basal membrane. Proteins like PC1, the NaK-ATPase β1 subunit, E-cadherin, and Sec6/8, accumulate in the cytoplasm of ADPKD cysts. However some proteins like alkaline phosphatase, leucine aminopeptidase and basal matrix receptors still have normal polarized distribution in ADPKD cystic tissue.

The specific pattern of membrane polarization in ADPKD cysts resemble the pattern observed in normal fetal renal tubular epithelia. The mispolarized proteins in

ADPKD are caused by failure of down-regulation of fetal pattern of gene expression in transcriptional level. PC1 may also directly be involved in the maintenance of membrane polarity, since PC1 participates in the formation of cell tight junctions. Tight junction can prevent the membrane mispolarization owing to the movement of proteins in the membrane plane.

Loss of membrane polarity in ADPKD leads to receptor, transporter and channel mislocalization and subsequently results in a proliferative and secretive phenotype. Therefore, loss of membrane polarity could be an important step after cystogenesis. Mutation of many other PKD genes also leads to alteration of membrane polarity.

1.3.3 Proliferation

Many factors contribute to the proliferative phenotype in ADPKD.

Epidermal growth factor receptor (EGFR) is up-regulated, dispolarized into apical membrane and heterodimerized with Her2/neu (ErbB2), a family membrane of EGFR. EGF and many other EGFR ligands are in a mitogenic concentration in cyst fluid. It has been shown that targeting EGFR attenuated PKD progression in several animal models.

The second messenger cyclic AMP (cAMP) possessess both proliferative and anti-proliferative capacity but it is cell type depdendent. In the normal kidney, cAMP is anti-proliferative. In ADPKD and ARPKD intracellular level of cAMP is up-regulated and it exerts strong proliferative effects because of reduced intracellular calcium level. The intracellular cAMP level is regulated by hormones such as vasopressin, parathyroid hormone, and calcitonin on different segment of nephron.

High level of IL-6, IL-8, colony stimulating factor (CSF) and vascular growth factor (VEGF) are accumulated in cysts of long-term dialysis patients, suggesting they may be involved in the expansion of cysts in PKD.

Polycystins may play a direct role in tubular cell proliferation, cell cycle and cell growth in PKD. The intracellular portion of PC1 interacte and sequester transcription factors STAT6 [6]. PC2 interact with Id2, a helix-loop-helix protein in a PC1 dependent manner and prevent its translocation to nucleus and suppression of p21waf, a cell cycle inhibitor, thus preventing cyclin-dependent kinase 2 (Cdk2) activation and cell cycle progression. The intracellular C-terminal tail of PC1 interacts with tuberin and its substrate mTOR, thus suppressing protein synthesis. PC2 interacts with pancreatic eIF2a kinase (PERK) in endoplasmic reticulum and enhances PERK dependent phophorylation of transcription initiation factor eIF2a.

1.3.4 Apoptosis

Apoptosis is the programmed cell death that is characterized by reduced cell volume, condensed chromosome, cell surface blebbing, and DNA cleavage leading to apoptotic bodies. Apoptosis is important for organogenesis since cell number need to be tightly regulated. It has been shown apoptosis is also enhanced in ADPKD and ARPKD especially, in non-cystic tubules, suggesting that cyst expansion is accompanied by the loss of normal tubules [2]. Therefore cyst formation in PKD resembles the process of organogenesis/tissue remodelling which needs not only a high rate of cell proliferation, but also a high rate of cell apoptosis. Although the compression of renal cysts may cause non-cystic tubular death, only 1-5% of nephrons are cystic in ADPKD. Therefore, it is plausible to conceive that apoptosis contributes to the renal failure in PKD.

Apoptosis has two pathways: one is intrinsic mitochondrial pathway, the other one is death receptor ligand mediated pathway [1]. The family of cysteine proteases, the caspase, plays a crucial role in promotion of apoptosis in both pathways. Recent studies suggest both pathways are responsible for apoptosis in PKD. The mRNA of FasL is not up-regulated in PKD, suggesting the death receptor pathway in PKD is independent of death receptor ligand FasL [1]. Several studies suggest TNF-α could a candidate ligand to stimulate apoptosis in ADPKD. It has been shown that PC1 conveys resistance to apoptosis of renal cell lines *in vitro*, suggesting a direct role of PC1 on apoptosis in PKD.

1.3.5 Secretion

Normal kidney is a net absorptive organ to regulate the balance of fluid and electrolyte. Mutation in PKD genes generates a secretary phenotype in the kidney and theraby stimulates cyst expansion. In ADPKD, the net secretion is caused by the disrupted sodium gradient, increased chloride excretion and increased activity of water channels [2].

In normal kidney, the low intracellular sodium level/sodium gradient is established by basal membrane localized NaK-ATPase. Sodium chloride enters apical membrane through the epithelial sodium and chloride channels. In ADPKD NaK-ATPase mispolarized into the apical membrane and pumping sodium and fluid into the cyst lumen. The mispolarization of NaK-ATPase is due to the failure of down-regulation of the fetal β2 subunit isoform of NaK-ATPase, favouring the assembly a1β2 Nak-ATPase heterodimers and its trafficking to apical membrane.

In ADPKD, chloride enters cells across basal membrane via NaK_2Cl cotransporters, driven by sodium gradient, and exits across apical membrane via

cAMP-dependent chloride transporter, cystic fibrosis transmembrane conductance (CFTR). Increased cAMP in ADPKD stimulates CFTR, which is highly expressed and functional on the apical membrane of ADPKD cystic tissue. Chloride secretion in ADPKD can also be enhanced by ATP-mediated chloride conductance. It has been shown that ADPKD epithelia can excrete more ATP and the truncated PC1 promote ATP-mediated chloride conductance.

Water enters cells/lumen across renal epithelial membrane via Aquaporin (AQP) water channels. AQP1 and AQP2 are highly expressed in ADPKD cystic epithelia and responsible for the fluid release.

The mechanism of fluid secretion is different between ADPKD and ARPKD. Although NaK-ATPase is also mispolarized in ARPKD cysts, it is either not functional, or its effects are counterbalanced by the resorptive activity of other sodium transporters. The role of CFTR in fluid secretion in ARPKD is also not clear.

1.3.6 Cell matrix interaction

One of the most prominent phenomenons of PKD is the thickened basement membrane and abnormal extracellular matrix (ECM) surrounding the cysts. Abnormal ECM in PKD was revealed by the accumulation of structural (collagen I and III, laminin) and soluble ECM-associated proteins (TGF-β, βigH3, periostin) [6]. The alteration in ADPKD basement membrane and extracellular matrix (ECM) is caused by the disrupted balance of matrix-degrading enzyme metalloproteinases and their specific inhibitors [21]. In addition, ADPKD and ARPKD epithelia were found to be avidly adhered to basement membrane than normal renal epithelia [22]. It was also found that ADPKD epithelia have poor migratory capacity [23]. All these suggest that there is a turnover defect of normal focal adhesion in ADPKD and ARPKD.

The high expression of fetal PC1 in punctate focal-adhesion-like bodies suggests a role of PC1 in the cell-matrix interaction that is necessary during early stage of renal development. In this early stage PC1 was required to coordinate the adhesion and migration of ureteric bud from underlying collagen matrix to undergo regulated branching morphogenesis. Many PKD proteins were found to be localized in focal adhesions. Disruption of components in focal adhesion complex leads to cystic kidney diseases.

Some ECM components like periostin and laminin contribute to the epithelial cell proliferation and cyst growth [6]. Periostin binds to α_v-integrins, which is highly expressed on ADPKD cysts, and leads to accumulation of nuclear β-catenin and Akt activation, two factors involved in cell proliferation. In vitro and in vivo studies showed that laminin-5 stimulate cell proliferation and cyst formation [6].

1.3.7 Cell-cell interaction

Early studies showed there were no abnormalities in cell-cell junction by showing the normal number and structure of tight junctions [2]. However recent molecular biology studies showed that E-cadherin, the normal component of cell-cell junction, is replaced by fetal isoform N-cadherin, which provide a poor differentiated scaffold for protein interaction between β-catenin and other actin-binding proteins [24]. Claudin-2, one of the components of tight junction in proximal tubules, is absent from the cysts [6]. Morever over-expression of β-catenin leads to cystic kidney diseases. Furthermore many PKD proteins are localized in cell-cell junctions.

The tight cell-cell junction is very important for the integrity and differentiated status of cells by preventing the dislocalization of polarized membrane proteins. Loss of differentiated status of cyst-ling epithelial cells can be observed from its

morphology. The morphology of ADPKD cyst-lining epithelia cells range from columnar to cuboidal to flattened, reflecting its segmental origin [2]. In the late stage of ADPKD, increased flattened cells were detected in cyst-lining epithelia, indicating the loss of differentiated structure.

1.3.8 Tubulointerstitium

Besides increased ECM, the abnormalities of vasculature, fibrosis and inflammation were observed in PKD [2]. It is not clear whether this is the secondary effect of maldevelopment of tubules or a direct effect of mutation of PKD genes.

Cyst expansion could generate local pericystic hypoxia in ADPKD and further up-regulate the production of hypoxia-inducible transcription factors (HIFs), which is independent of PKD (Eckardt 2007). The extensive/abnormal vasculature detected in the ADPKD cyst walls could be direct result of hypoxia mediated vasculogenesis [25, 26].

Histology studies have shown that the degree of fibrosis in ADPKD patients is associated with the severity of disease [27]. Fibrosis can be caused by dysregulation of matrix proteins, peritulular capillaries and activation of fibroblasts [28]. Hyperproliferation of fibrosis has been detected in ADPKD as a result of increased production of acidic-fibroblast-growth-factor [29]. Some fibroblasts in PKD may be derived from tubular epithelial cells via epithelial-mesenchymal transformation (EMT) [30].

In addition to fibrosis, the degree of inflammation is also associated with the progress of chronic renal disease, but attracts less attention in the field of PKD. Increased expression of proinflammatory factors like monocyte chemoattractant protein-1 (MCP-1) and osteopotin were detected in the rat model of ADPKD. NFkB, the most important proinflammatory transcription factor, is increased in PKD-deleted

HEK293 cells and PKD-1 mutated cystic cells, contributing to cell proliferation and survival [31]. The proinflammatory factor TNF-α is also overexpressed in cystic tissues and plays a role in cystogenesis [15].

1.4 Hypothesis of cystogenesis

1.4.1 Cilia pathology

It is currently accepted that PKD is a disorder of primary cilia [18]. Most PKD proteins locate in primary cilia of renal epithelial cells. Inactivation of ciliogenic genes like *Kif3a* and *Ift88*, leads to cystic kidney diseases.

The primary cilium has hair-like structure and is found in most cell surfaces. It has a bundle of microtubules, called axoneme, surrounded by cell membrane. Primary cilium is anchored in basal body, which functions as centriole during mitosis. Assembly, maintenance and function of cilia require the movement of proteins along ciliary microtubules, a process called intraflagellar transport (IFT). Disruption of IFT genes prompted cystogenesis in animal models. Renal epithelial primary cilia function as mechanosensors of urine flow. Fluid flow bends primary cilia and induces calcium entry by mechnosensitive PC2 calcium channel [9].

Mutation of PKD genes impairs primary cilium functions leading to disrupted planar cell polarity and initiate cyst formation [18]. Dysfunction of primary cilia in PKD leads to a low intracellular calcium level, which generates a cAMP-dependent proliferative phenotype.

1.4.2 Gene dosage

Although ADPKD is inherited as a dominant trait, it was thought be transmitted in a recessive fashion at molecular level. The two hit hypothesis suggests a second mutation in the normal allele of ADPKD leading to cystogenesis. However it has been shown that induction of cyst formation does not need the complete loss of PKD genes. 70-80% reduction of pkhd1, pkd1 gene, leads to cyst formation [5, 32-34]. However overexpression of *pkd1* and *pkd2* genes also leads to cystogenesis, suggesting the correct PKD gene dosage is essential for normal renal morphogenesis and the absence of cystic diseases [6]. Recent studies showed that centrosomal amplification occurs in kidneys from PKD gene knocking out, over-expressing animals and from ADPKD patients [35, 36]. The link between dysregulated PKD genes, centrosomal amplification and genetic instability may reconcile the haploinsufficiency, gene dose effect, and two hit hypothesis.

1.4.3 Timing of cystogensis and non-genetic factors (third hit)

Conditional knocking out of ciliogenic or PKD genes in developing mouse kidneys leads to rapid development of PKD [6, 18]. Inactivation of these genes in adult mice only causes a slower progressive cyst disease, despite of comparable loss of cilia. These findings suggest that the timing of cystogenesis is critical and blocking cystogenesis in the early time could change the course of disease progression.

The difference between a developing kidney and mature/adult kidney is the high rate of proliferation and apoptosis, resembling the process that occurs during organogenesis and tissue remodelling upon injuries, infections and toxins [1, 18, 37]. Indeed renal ischemia/reperfusion injuries accelerate cyst formation in adult mice with mutation of ciliary genes [18]. In male ADPKD patients, urinary infection is

associated with worsening of renal function [38]. Moreover, it was once demonstrated in a mice model that cyst formation is significantly reduced in a germ-free environment compared to conventional conditions. Recent studies show that up-regulation of genes expressed in activated macrophages and complement system factors is associated with severe disease in *cpk* mouse model of ARPKD [39]. In addition, administration of TNF-a, a potent cytokine induced in acute kidney injury or infections, promotes cystogenesis in adult mice with single germline mutation of *PKD2* gene [18]. A new hypothesis has been proposed with says that a third hit (non-genetic/environmental factors) is required to initiate and accelerate cystogenesis [9]. Indeed a nice observation in adult *Pkd1* conditional knockout mouse shows that highly proliferating cells only present in cyst-lining and adjacent normal-looking but not distant epithelia, suggesting *Pkd1* inactivation is not enough to initiate cell proliferation and a local paracrine mechanism/chemical gradient may account for focal cell proliferation and regional disease progression [40]. Nevertheless *Pkd1* inactivation predisposes the tubular epithelial cells to a third hit, at which time the cell enters the cell cycle and cysts form.

Taken together, rapid formation of renal cysts following inactivation of ciliogenic or PKD genes requires a high rate of proliferation and apoptosis, which is present in the early life of developing kidneys or in disease conditions. It has been shown that early onset of ESRD in ADPKD patients attributes to the number of cysts formed in the early ages of life instead of cyst growth rate. The high intra-familiar variability in ADPKD patients could be probably explained by the individual variation in exposure to the environmental factors in their early ages.

1.5 Signaling in polycystic kidney disease

1.5.1 Mammalian target of rapamycin (mTOR)

Structure and function

Target of rapamcin (TOR) is a highly conserved kinase discovered from prokaryotes to eukaryotes [41]. It actively controls energy consuming anabolic processes (transcription, protein synthesis, ribosome biogenesis, nutrient transport and mitochondrial metabolism) and negatively regulates catabolic processes (mRNA degradation, ubiquitin-dependent proteolysis, autophagy and apoptosis).

mTOR is a member of the phosphoinositide 3-kinase related kinase (PIKK) family, whose catalytic domain has significant amino acid homology to that of the lipid kinase phosphoinositide 3-kinases (PI3Ks) [42]. Unlike other PIKK family members, mTOR contain a FRB (FKBP12-rapamycin binding) domain which is required for FKBP12/rapamycin binding and lays immediately N-terminal to the kinase domain.

mTOR is found in two functional and structurally distinct complexes: mTORC1 and mTORC2 [41]. The defining component of mTORC1 is rapamycin-sensitive adaptor protein of mTOR (raptor), whereas the core unit of mTORC2 is ramapycin-insensitive companion of mTOR (rictor).

mTOR integrates signals from nutrients, energy and growth factors. Growth factors activate mTORC1 via PI3K-Akt/PKB pathway. Energy regulates mTORC1 via adenosine monophosphate (AMP)-dependent protein kianse (AMPK). Akt inactivates but AMPK activates TSC1/2 complex, which serves as a hub to integrate signals from energy and growth factors. TSC1/2 complex inhibits mTORC1 activity through suppressing small GTPase Rheb. Nutrients are the dominant TOR input. High amino acid level can compensate the absence of other mTORC1 inputs but not vice versa. Amino acids activate Rag GTPase by increasing GTP loading. The activated Rag

binds mTORC1 and delivers it to growth factor or low energy-activated Rheb for further activation of mTORC1.

The most important substrates of mTORC1 are S6K and 4E-BP1. They both contain TOS motif to interact with Raptor [42]. Phosphorylation of 4E-BP by mTOR release the it`s repression on the transcription factor eIF4B, which bind the 5`-end of mRNAs. Active mTOR activates eIF4B-mediated mRNA translation. mTOR also phosphorylates but activates another stubstrate S6K, which is originally identified as the kinase of ribosomal protein S6 (rpS6). However S6K stimulate cell proliferation and growth pathway through several other downstream targets (eIF4b, eEF2K) besides rpS6.

Little is known about the uspstream regulation of mTORC2. Akt and serum glucocorticoid-induced kinase (SGK) are the two substrates of mTORC2 [43]. Previously mTORC2 is thought not sensitive to rapamycin, however it is found to be sensitive to rapamycin only in a high dose and long-time treatment [44].

There is a negative feedback loop from S6K where S6K can phosphorylate and inhibit IRS/PIK/Akt pathway.

mTOR signalings in PKD

mTOR is aberrantly activated in PKD; inhibition of mTOR retards disease progression in animal models of PKD [1]. PC1 inhibits mTOR activity probably by interacting with TSC2 and mTOR with its cytoplasmic tail [45]. Overexpression of growth factors and their receptors such as IGF-1, EGF, EGFR were observed in animal models of PKD and human ADPKD patients [2, 46]. Accumulation of these growth factors in the cyst kidney could stimulate Akt/mTOR pathway. Indeed Akt was found to be activated in Han:SPRD rat model of ADPKD [47].

1.5.2 Calcium/cAMP

Calcium and adenosine 3':5'-cyclic monophosphate (cAMP) are important secondary messengers and implicated in cystic disease. Defection of polycystins or fybrocystin disrupts the cytoplasmic membrane calcium channels and reduces the intracellular calcium stores in PKD [48]. Decreased intracellular calcium level stimulates calcium inhibitable adenylyl cyclise 6 and/or inhibits calcium-dependent phosphodiesterase 1, resulting in a high intracellular cAMP level. The disrupted intracellular calcium homeostasis switches cells from cAMP-dependent anti-proliferative to cAMP-dependent proliferative phenotype in PKD. In normal kidneys, cAMP inhibits the Ras/Raf-1/MEK/ERK pathway at the level of Raf-1, thus decreasing cell proliferation. In contrast, in conditions of calcium deprivation, cAMP stimulates B-Raf and activates MEK/ERK pathways in PKD, thus promoting cell proliferation [5].

1.5.3 Tumor necrosis factor-alpha (TNF-a)

Recent studies showed that Tumor necrosis factor-alpha (TNF-a) could be non-genetic factor initiating cystogenesis and accelerating disease progression [49]. TNF-a is characterized by its ability to stimulate cell apoptosis and proliferation. In disease conditions such as urinary infections, renal injuries and hypertensive stresses, TNF-a mRNA and protein are up-regulated [49]. Of note, TNF-a is up-regulated in mouse and human PKD. Inhibition of TNF-a and TNF-a-converting enzyme were shown to attenuate disease progression in mouse PKD models [15, 50]. TNF-a reduces the amount of PC2 transported to the plasma membrane and primary cilium via increasing the production of FIP2. FIP2 is an interacting protein which can

specifically interact with PC2 and prevents it trafficking to plasma membrane and primary cilium.

TNF-a can stimulate proliferation via activating IKKb, which interacts with and suppresses the activity of TSC1/TSC2, thus activating the downstream mTOR pathway [51]. On the other hand, TNF-a may induce apoptosis via the death receptor mediated pathway [2, 52].

Taken together TNF-a may initiate cyst formation by reducing the dosage of PC2 in primary cilium and creating a focal microenviroment of high cell proliferation and apoptosis rate.

1.6 Diagnosis

Although the relative low sensitivity, ultrasound is still the most common tool to diagnose ADPKD due to the cost and safety reasons. The ultrasonographic criteria for ADPKD are based on the patient`s age, family history, and number of cysts [6, 8]. The image resolution can be increased up to four times with magnetic resonance imaging (MRI) or computed tomography (CT), which can detect a minimum three millimetre cysts. The MRI technique is believed to be better than CT because of the repeated exposure of ionizing radiation to ADPKD patients during CT scan. Advances of molecular genetics and identification of PKD genes allows diagnosis to be further confirmed by DNA sequencing. Although using current techniques can detect 90% of mutations, classification of these mutations as pathogenic or just harmless polymorphism is still difficult. For ARPKD, the large *PKHD1* gene, high level of allelic heterogeneity and large number of spliced variants make molecular diagnosis very difficult.

1.7 Animal and cell culture models of polycystic kidney disease

Numerous murine models phenotypically closely resemble human PKD with respect to cyst morphology, cyst location and disease progression. Mutation in these murine models can be spontaneous, chemical or genetic engineered [53].

1.7.1 Rat model

Only few rat models of PKD are available and they are all spontaneous mutants [53]. Heterozygous cystic (Cy/+) Han:SPRD rat is a well characterized and extensive studied model for ADPKD. It resembles human ADPKD phenotypically and transmits the disease in an autosomal dominant fashion. The mutation was found in 1989 in Hanover in a Sprague-Dawley rat strain [54]. Few cystic lesions can be observed during the first four weeks of life in Cy/+ Han:SPRD rats and develop aggressively between week 4 to week 8 [54]. Kidney enlargement can be observed already during first 2-3 weeks and increases rapidly until week 8 (2 kidney weight up to 8 gram). Kidney size grows until week 12 and regress from week 12. It becomes stable between week 24 (2 kidney weight around 4.8 gram) to week 44 although still significantly higher than +/+ control rats. Significant azotemia develops in Cy/+ rats at week 8 and progresses continuous thereafter. Like human ADPKD, there is gender dimorphism in Cy/+ rats. Life span of male Cy/+ rats is between 10-12 months. Female heterozygotes have a normal life span, which is around 2 years. The basement thickness is observed at week 2. But significant inflammation is only observed until week 24.

Cy/Cy homozygotes develop a rapidly progressive form of PKD with massive renal enlargement and rapid-onset of azotemia and death by week 3. Cystic changes involves in the whole nephron segment.

The limitation of this rat model is that cysts only develop in the proximal tubule in Cy/+ rats and they have no extrarenal manifestation, which are not similar to the human ADPKD.

Mutation in *Pkdr1* in chromosome 5 causes the disease in Han:SPRD rats. The gene *Pkdr1* encodes a transmembrane protein samcystin, but its function remains elusive.

The Wistar polycystic kidneys (*wpk*) mutant develops spontaneously in Wistar rat strain and is inherited in a recessive trait. Cysts are initiated at E19 in the proximal tubules, thick limbs, distal tubules and collecting ducts in homozygotes. Cystic changes progressively shift predominantly to collecting ducts and result in death at week 4. *Wpk* gene is not well characterized. *Wpk* rats are regarded as a ARPKD model, however, the biliary ductal plate malformation is not evident in this model.

The mutation of polycystic kidneys (pck) arises spontaneously in Crj:CD/SD rat strain and is transmitted in a recessive fashion. Cystic lesions are observed in thick ascending limb of Henle, distal tubules and collecting ducts after 1 week of age in homozygotes. The cystic changes progress slowly and exhibit focal interstitial inflammation and fibrosis by 70 days of age. Biliary duct dilation is observed as early as 1 day of age. Male *pck* rats develop disease more severe than the females. Given the late-onset and slowly progressive PKD, the *pck* rats was initially regarded as a model of ADPKD. However it was found that the gene *Pkhd1*, orthologe of human ARPKD gene, was mutated in this rat strain.

1.7.2 Mouse model

Most mouse PKD models inherit disease in a recessive trait. Several models resemble ARPKD with respect to cyst pathology and disease progression. Others

have cysts distributed along the whole nephron, have extrarenal manifestation, progress slowly, and therefore are more closely approximate to human ADPKD phenotype.

Spontaneous model

The first described and most extensive studied mouse PKD model is congenital polycystic kidneys (*cpk*) mouse. The mutation arises spontaneously in the C57BL/6J (B6) mouse strain. The disease is transmitted as a recessive trait and phenotypically resembles ARPKD. Cystic changes are initiated at embryonic day 16 (E16) and locate primarily in proximal tubule, but progressively shift predominantly to collecting ducts after birth. Death occurs by 3-4 weeks of age.

The disease expression and severity can be modulated by genetic background. *cpk* genes locate in chromosome 12 and encodes cystin, which is localized to the primary cilium.

The BALB/c polycystic kidneys (*bpk*) mouse and the inversion of embryonic turning (*inv*) mouse are proposed as ARPKD model, with 4 week and 1 week life span respectively.

The juvenile cystic kidney (*jck*) mouse, the kidney, anemia, testes (*kat*) mice and polycystic kidney disease (*pcy*) mouse have ADPKD phenotype, with 4 months, 2 months and 8 months of life span respectively. Of note, the gene mutated in *pcy* mouse is the orthologe of the human *NPHP3* gene. Therefore *pcy* mouse may be a more appropriate model for the human NPH/MCD complex than ADPKD.

Spontaneous model with immune system involvement

There are two PKD mouse models with intensive immune system involvement, although the topic is presently out of focus in the field of PKD.

The spontaneous *CFWwd* mutant is inherited in autosomal dominant trait and displays ADPKD phenotype with respect to cyst morphology and extrarenal manifestations. But the penetrance of ADPKD phenotype is strongly influenced by environmental exposure. In the conventional facility, 100% of *CFWwd* mouse developed the disease, while only 4% of *CFWwd* mouse develop disease under germ-free conditions. The responsible gene for *CFWwd* mouse was not identified.

kd mouse is inherited as recessive trait and develops T cell-mediated autoimmune interstitial nephritis and die at 5-7 months. Cystic dilation with focal peritubular mononuclear cell infiltration is observed in renal cortex between 10-14 weeks of age. The *kd* mouse histopathologically resembles NPH/MCD disorders. The responsible gene for *kd* mouse is not identified.

Chemical induced model

The jcpk mutant was discovered in a chlorambucil mutagenesis program and phenotypically resembles ADPKD. Interestingly *jcpk* is allelic to *bpk*. Probably the gene product of *jcpk* is less functional than *bpk* gene product.

Genetic model

A large number of PKD mouse models with null or hypomorphic alleles of *pkd1* and *pkd2* orthologes have been generated [9, 53]. Homozygous mutation of PKD genes mostly leads to embryonic death in animals. Heterozygous mutation (50% reduction of gene dosage) of PKD genes does not lead to cystogenesis or induces very milder tubular dilation. Very recently it has been shown that double mutation of pkd1 genes in new born mice generated a faster cystic growth phenotype. Data from these *pkd1* and *pkd2* deficient mouse support two-hit hypothesis. However overepression of *pkd1* and *pkd2* genes also generate an ADPKD phenotype in mice.

1.7.3 Cell culture model

With primary renal epithelial cells from ADPKD and ARPKD patients, Yamaguchi *et al* discovered the mechanism of proliferative phenotype of cAMP in PKD [55]. Transfection of the dog renal epithelial cell line MDCK cells with *pkd1* gene or using MDCK cells for 3D culture advanced our knowledge about the pathogenesis of PKD proteins [56, 57].

1.8 Therapies

Current therapy for PKD is directed towards limiting its morbidity and mortality from the complications of this disease [6, 8, 18]. PKD patients are managed with supportive treatments to control pains, hypertension, and cyst infections. They are recommended to avoid caffeine and estrogens. Renal replacement (transplantation or dialysis) therapy is required for PKD patients in ESRD.

Molecular advances in the mechanism of PKD and availability of more animal models facilitate designing new preclinical trials. Since the onset of cyst growth precedes the loss of renal function, current belief by experts in this field is that retardation of cyst growth will ultimately improve the loss of renal function. Several recent studies on PKD are underway in clinic and in animal models with drugs targeting signaling pathways which result in aberrant cell proliferation, apoptosis and fluid secretion.

1.8.1 mTOR inhibition

Rapamycin (or sirolimus, the official generic name), the first discovered mTOR inhibitor, is a macrocyclic lactone and found in the soil bacterium *Strpomyces hygroscopicus* on Easter Island [1, 58]. Everolimus is an oral 40-O-(2-hydroxyethyl) derivative (not prodrug) of sirolimus and has a shorter half-life as compared with sirolimus [58, 59]. Rapamycin/everolimus inhibit mTOR activity by forming a complex with its intracellular receptor FKBP12 and in turn interacting with the FRB domain of mTOR.

Sirolimus/everolimus was approved by Food and Drug Administration (FDS) to prevent graft rejection in the kidney transplant patients and inhibits tumor growth in cancer patients, although mild adverse effects were observed in clinical trials [1].

mTOR inhibition by sirolimus or everolimus demonstrates promising results in several animal models of PKD and in transplant ADPKD patients. Sirolimus or everolimus retarded or even reversed cyst growth and disease progression in several rat and mouse models of PKD [45, 60-63]. In renal transplant patients, sirolimus reduced the size of renal and liver cysts in renal transplant patients [45, 64]. The clinical trial in PKD patients with everolimus trial has been initiated recently; however animal studies with everolimus on its efficacy and safety are still limited.

1.8.2 Inhibition of other mitogeneic pathways

Because of the importance of calcium and cAMP in PKD, targeting calcium or cAMP may inhibit cell proliferation and cyst growth in PKD. Increased water intake, vasopressin V2 receptor (VPV2R) antagonists and somatostatin inhibit cAMP-mediated cystogenesis by reducing vasopressin level, VPV2R activity and activating SST2 receptor, respectively [6]. Indeed a small scale clinical trial showed that 6-month

somatostatin therapy dccreases further the increase of cyst volume in ADPKD patients [65]. Correcting intracellular calcium haemostasis by triptolide also reduced cystic burden in *Pkd1* knockout mice [6]. Targeting MAPK or mTOR, downstream of cAMP signaling, inhibited cell proliferation and cyst development in PKD animals [6, 66]. Inhibition of cell cycle progression by roscovitine arrested cyst growth in two mouse PKD models [67]. Many arachidonic metabolites have mitogenic properties. Inhibition of these metabolites reduces cyst formation in vitro and in vivo [6].

1.8.3 Inhibition of fluid secretion

In order to inhibit fluid secretion-driven cyst expansion in PKD, transporters (NaK2Cl cotransporter, Na-K-ATPase, CFTR and KCa3.1) required for chloride secretion have been targeted. CFTR inhibiters were administered in mouse models of PKD, directly inhibited chloride secretion and therefore slowed cyst growth [68]. Targeting KCa3.1 *in vitro* showed promising data in inhibiting chloride secretion and cyst formation. However targeting NaK2Cl cotransporter and Na-K-ATPase should be done cautiously since severe side effects can be induced [6].

1.8.4 Targeting hypertension

Hypertension occurs before and after the loss of renal function in ADPKD and may be attributed to the cyst compression-mediated activation of renin-angiotensin-aldosterone system (RAAS) [69]. RAAS may also play a multifactorial role in the pathogenesis of cyst growth by enhancing apoptosis, proliferation, fibrosis and production of pro-inflammatory factors. Thus, targeting RAAS by drugs like rennin inhibitors, ACEIs, angiotensin receptor blockers (ARBs), or adosterone antagonists may provide reno-protective effects in ADPKD patients.

1.8.5 Targeting angiogenesis, apoptosis, and inflammation

The role of VEGF in PKD is not conclusive. Inhibition of angiogenesis by VEGFR inhibitors showed beneficial effects in two different animal models of PKD [70, 71]. However early inhibition of VEGFR2 during perinatal period in CD-1 mice results in renal cyst formation [72].

The progression of PKD can be retarded by targeting apoptosis and inflammation. However the mechanism is still unknown. Inhibition of Caspase or specific deletion of caspase-3 can slow disease progression and prolong life in animal models of PKD [73, 74]. Treatment with TNF-α inhibitor etanercept in *Pkd2* mutant mice reduced cyst formation via restoring the transport of PC2 to primary cilium. Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists improved embryo survival and renal/extrarenal abnormalities in *pkd1* knockout mice, probably via affecting Wnt β-catenin signalings.

Chapter 2

Everolimus Retards Cyst Growth and Preserves Kidney Function in a Rodent Model for Polycystic Kidney Disease

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Everolimus Retards Cyst Growth and Preserves Kidney Function in a Rodent Model for Polycystic Kidney Disease

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Key Words
Polycystic kidney disease · Everolimus · mTOR · Han:SPRD rat

Abstract
Background/Aims: Rapamycin inhibits cyst growth in polycystic kidney disease by targeting the mammalian target of rapamycin (mTOR). To determine if this is a class effect of the mTOR inhibitors, we examined the effect of everolimus, the analogue of rapamycin, on disease progression in the Han:SPRD rat model of polycystic kidney disease. ***Methods:*** Four-week-old male heterozygous cystic (Cy/+) and wild-type normal (+/+) Han:SPRD rats were administered everolimus or vehicle (3 mg/kg/day) by gavage for 5 weeks. Kidney function and whole-blood trough levels of everolimus were monitored. After treatment kidney weight and cyst volume density were assessed. Tubule epithelial cell proliferation was assessed by BrdU staining. ***Results:*** Everolimus trough levels between 5 and 7 μg/l were sufficient to significantly reduce kidney and cyst volume density by approximately 50 and 40%, respectively. The steady decrease of kidney function in Cy/+ rats was reduced by 30% compared with vehicle-treated Cy/+ rats. Everolimus treatment markedly reduced the number of 5-bromo-2-deoxyuridine-labeled nuclei in cyst epithelia. Body weight gain and kidney function were impaired in everolimus-treated wild-type rats. ***Conclusion:*** Moderate dosage of everolimus inhibits cystogenesis in Han:SPRD rats. The inhibitory effect of everolimus appears to represent a class effect of mTOR inhibitors.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the leading causes of end-stage kidney disease and occurs in 1–2 per 1,000 live births [1, 2]. The disease is characterized by aberrant proliferation of tubular epithelial cells (TEC) forming multiple renal cysts [1]. Currently there is no specific treatment for ADPKD other than supportive care, and dialytic treatment or renal transplantation when patients have reached end-stage renal disease. Because ADPKD progresses slowly, there is a window of opportunity to treat the disease by using therapeutical principles which retard cystic expansion, thus preventing the development of end-stage renal disease.

The mammalian target of rapamycin (mTOR), an atypical serine/threonine kinase, is a central controller of cell growth and proliferation [3]. The mTOR pathway is not activated in normal adult kidneys but is aberrantly activated in kidneys of different rodent models for polycystic kidney disease (PKD) as well as in cyst-lining cells in human ADPKD [4, 6]. Rapamycin (also termed siroli-

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mus) is a specific inhibitor of mTOR with strong antiproliferative properties. Rapamycin is clinically used to prevent organ rejection in renal transplant patients. Furthermore, cardiac stents coated with rapamycin abolish intrastent neointimal proliferation. Recently we and others have shown that rapamycin when administered intraperitoneally or through the drinking water successfully retards disease progression in different rodent models of PKD [5–7]. Relatively low blood level of rapamycin decreased cyst volume density by approximately 20% in the Han:SPRD rat, a model for PKD [6].

Everolimus [40-O-(2-hydroxyethyl)-rapamycin], also called RAD001, is a macrolide antibiotic that binds to the intracellular protein FKBP and thereby inhibits mTOR activity [8]. It is an orally active derivative of sirolimus (rapamycin), with a hydrophilic 2-hydroxyethyl chain at position 40 [9]. Compared to rapamycin, everolimus has a shorter half-life and a higher biological availability [10]. Everolimus is also used as an immunosuppressive drug in organ transplantation. Currently an ongoing clinical trial is testing whether everolimus (Certican®; NCT00414440) whole-blood trough levels between 3 and 8 μg/l halt disease progression in human ADPKD. Surprisingly, it is not known if everolimus halts disease progression in PKD animal models.

Han:SPRD rats represent one of the most commonly used rodent models of PKD, phenotypically resembling human ADPKD [11]. To determine whether the observed cyst inhibition is rapamycin-specific or if this is a class effect of the mTOR inhibitors, we examined the effect of everolimus on renal functional loss and cyst progression in the Han:SPRD rat model of ADPKD. To achieve consistent blood levels of everolimus, we employed daily gavage feeding in this study.

Materials and Methods

Animals

This study was conducted in heterozygous cystic (Cy/+) and wild-type normal (+/+) rats. Only male rats were used, since cysts develop more rapidly in male than female rats [12]. The Han:SPRD rat colony was established in our animal facility from a litter which was obtained from the Rat Resource and Research Center (Columbia, Mo., USA). The study protocol was approved by the regulatory commission for animal studies, a local government agency. Rats had free access to tap water and standard rat diet.

Study Drug

Everolimus microemulsion (20 mg/g) and vehicle microemulsion were kindly supplied by Novartis (Basel, Switzerland) and stored at –20°C. Everolimus and vehicle were diluted with tap water before oral administration.

Experimental Protocol

Male Cy/+ and +/+ rats were weaned and then treated with 3 mg/kg/day everolimus (Cy/+, n = 8; +/+, n = 10) or vehicle (Cy/+, n = 4; +/+, n = 11) at 4 weeks of age. Everolimus was administered once daily by gavage feeding for 5 weeks (day 0 to day 35) at a volume of 2 ml/day. The dose of everolimus administered was adjusted daily according to the rat body weight.

Tail blood was obtained from rats on days 0, 17 and 36. Whole blood for everolimus levels and plasma for blood urea nitrogen (BUN) were stored at –20°C. Blood everolimus levels and BUN were analyzed later by kinetic color test and HPLC mass spectroscopy at the Institute for Clinical Chemistry, University Hospital (Zürich, Switzerland). Everolimus 6-hour levels were measured on day 17 and everolimus trough levels (24-hour level) on day 36. BUN concentrations were determined on days 0, 17 and 36.

For the assessment of cell proliferation 5-bromo-2-deoxyuridine (BrdU) (Sigma, St. Louis, Mo., USA) was injected subcutaneously (20 mg/kg/day BrdU in 0.9% NaCl) in 3 rats per group on days 33, 34 and 35.

After the 5-week treatment the rats were anesthetized with isoflurane, and kidneys were excised, decapsulated and weighed.

For histologic examinations, 3 rats per group were anesthetized by an intraperitoneal injection of pentobarbital (100 mg/kg body weight) and fixed by vascular perfusion [13]. The fixative contained 3% paraformaldehyde (PFA), 0.01% glutardialdehyde and 0.5% picric acid, dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, added with sucrose, final osmolality 300 mosmol) and 4% hydroxyl ethyl starch (Fresenius Kabi, Bad Homburg, Germany) in 0.9% NaCl. The kidneys were fixed for 5 min and then rinsed by vascular perfusion with 0.1 M cacodylate buffer for 5 min.

Histology

Two- to three-millimeter-thick mid-transversal rat kidney slices were dehydrated in ethanol series, embedded in paraffin and subjected to periodic acid-Schiff staining for cyst volume density determination.

Cyst volume density was assessed by morphometry, using the method of point counting [14]. For morphometry 4–6 micrographs were obtained for 1 section per rat as described before [6]. The pixel size of the micrographs was 1,300 × 1,030 (1.34 μm/pixel). An orthogonal grid with a line spacing of 150 pixels was used. The reviewer who performed the morphometry was blinded to experimental groups.

Anti-BrdU clone 3D4 (BD Biosciences Pharmingen, San Diego, Calif., USA) was mouse monoclonal. For the detection of BrdU, 2-mm-thick slices of perfusion-fixed kidney were frozen in liquid propane cooled down to –196°C by liquid nitrogen and cut into 4-μm-thick cryostat sections. The cryostat sections were microwaved for 10 min in 0.01 M citrate buffer at pH 6.0. After pretreatment in 5% normal goat serum in PBS, the cryostat sections were incubated overnight in a humidified chamber at 4°C with the primary antibody, diluted in PBS-1% BSA. Binding sites of the primary antibodies were revealed with a fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). For staining of brush border in the proximal tubule, rabbit-anti-mXT2 [targeting the 18 amino acid peptide antigen NH_2-MAQ ASG MDP LVD IED ERC-COOH; affinity purified, dilution 1:200] was applied and followed by the secondary antibody Alexa Fluor 594 donkey anti-

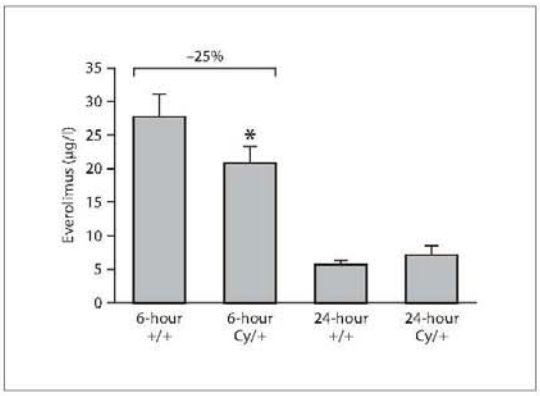


Fig. 1. Whole-blood everolimus levels in male Cy/+ and +/+ rats. Whole-blood 6- and 24-hour trough levels of everolimus were obtained in all everolimus-treated rats. The everolimus 6- and 24-hour trough levels were 27.7 ± 3.1 and 5.8 ± 0.4 µg/l in +/+ rats, and 20.7 ± 2.4 and 7.1 ± 1.2 µg/l in Cy/+ rats. * $p < 0.05$ Cy/+ at 6 h versus +/+ at 6 h.

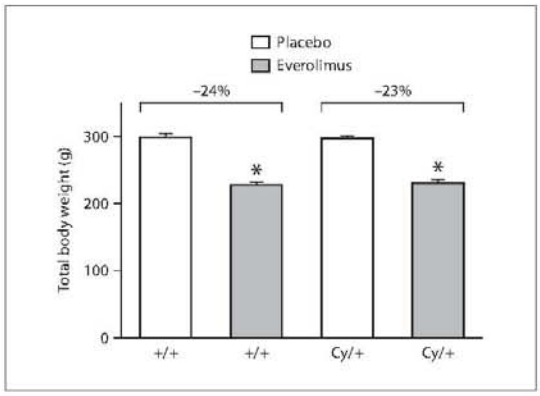
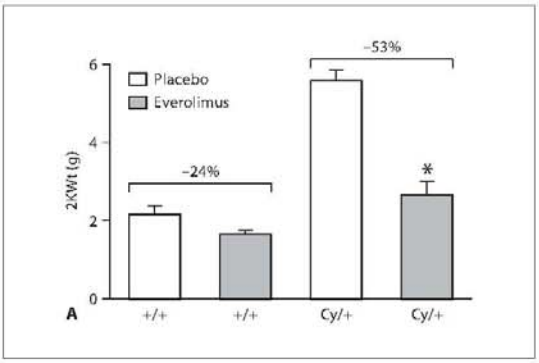


Fig. 3. Everolimus retards body weight gain in rats. After 5 weeks of everolimus treatment (day 36) the body weight gain of +/+ and Cy/+ rats was significantly retarded by 24 and 23%, respectively (ANOVA). * $p < 0.001$ +/+ or Cy/+ with vehicle versus +/+ or Cy/+ with everolimus group.



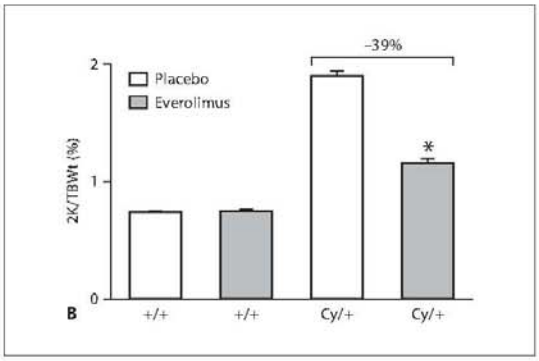


Fig. 2. Everolimus retards the increase in kidney weight in Cy/+ rats. **A** Two-kidney weight (2KWt) of Cy/+ rats was reduced by 53% after everolimus treatment. **B** Two-kidney/total body weight (2K/TBW) of Cy/+ rats was reduced by 39% after everolimus treatment. * $p < 0.001$ Cy/+ with vehicle versus Cy/+ with everolimus group.

rabbit IgG (1:1,000; Molecular Probes, Portland, Oreg., USA) [15]. For control of unspecific binding of secondary antibodies we made control incubations by omitting the primary antibody. These control experiments were negative. The sections were placed on coverslips, using DAKO-Glycergel (Dakopatts), to which 2.5% 1,4-diazabicyclo[2,2,2]-octane (DABCO; Sigma, St. Louis, Mo., USA) was added as fading retardant. Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung, Vienna, Austria). The fluorescence for Alexa Fluor 594 and fluorescein isothiocyanate were recorded separately using U1 and B2 filter modules, respectively. Overlays of the 3 channels were performed with the Image-Pro software. Images were acquired with a charge-coupled device camera (Visicam 1280, Visitron Systems, Puching, Germany) and processed by Image-Pro and Photoshop software.

Statistical Analyses

Statistical analyses were performed by one-way ANOVA with the Newman-Keul post hoc test using GraphPad Prism 4 software. All data are expressed as means ± SEM and $p < 0.05$ was considered as statistically significant.

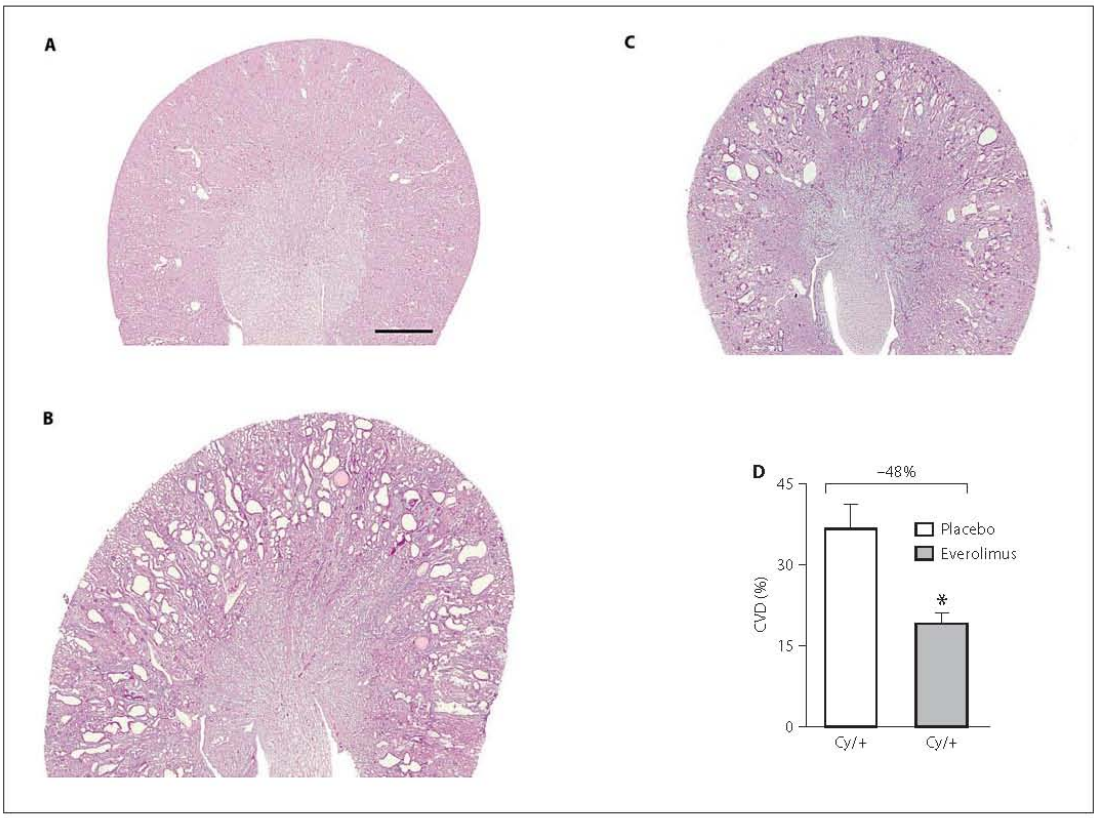


Fig. 4. Everolimus decreases the renal cyst growth in Cy/+ rats. Representative paraffin-embedded kidney sections of wild-type/vehicle (+/+; **A**), heterozygous/vehicle (Cy/+; **B**), heterozygous/everolimus rat (Cy/+; **C**). **D** Cyst volume density (CVD) of Cy/+ rat was reduced by 48% after everolimus treatment. * $p < 0.001$ Cy/+ with vehicle versus Cy/+ with everolimus group. Bar scale: 1 mm.

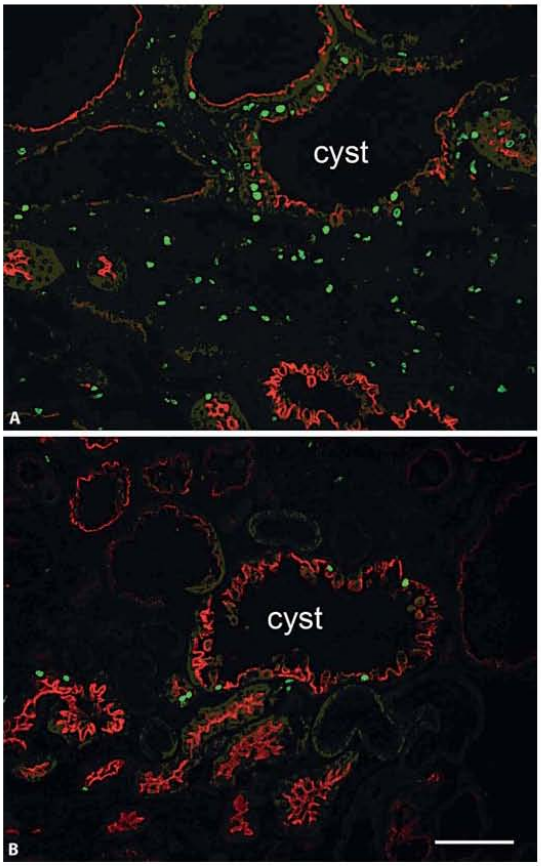
Results

Blood Level of Everolimus

The everolimus 6-hour and 24-hour trough whole-blood levels were 27.7 ± 3.1 and 5.8 ± 0.4 μg/l in +/+ rats, and 20.7 ± 2.4 and 7.1 ± 1.2 μg/l in Cy/+ rats (fig. 1). The lower everolimus blood levels at 6 h in Cy/+ rats (25%, $p < 0.05$) compared with +/+ rats suggest a difference in absorption and/or metabolism. The difference of everolimus trough levels between +/+ and Cy/+ rats was not significant.

Everolimus Treatment Retarded the Increase of Kidney Weight

The once a day administration of everolimus by gavage feeding for 5 weeks reduced 2-kidney weight in Cy/+ Han:SPRD rats by 53% ($p < 0.001$; fig. 2A). Figure 2B shows that everolimus reduced the 2-kidney/total body weight ratio by 39% ($p < 0.001$) in Cy/+ rats. Everolimus treatment had no effect on the 2-kidney/total body weight of +/+ rats. Everolimus treatment lowered body weight gain in the same magnitude in +/+ (n = 10) and Cy/+ (n = 8) rats. The mean weight difference was –24% for +/+ ($p < 0.001$) and –23% for Cy/+ rats ($p < 0.001$) on day 36 compared to vehicle-treated rats (fig. 3). These results



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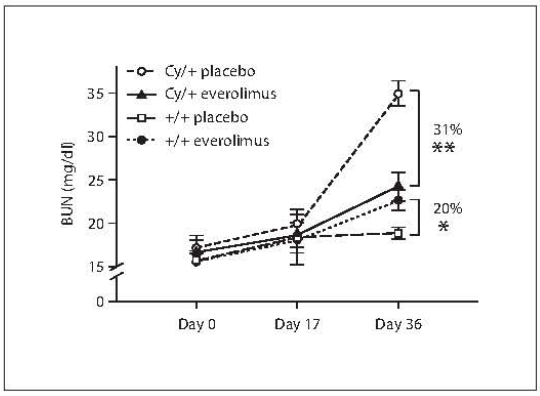

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Fig. 5. Proliferating cells are reduced after everolimus treatment in Cy/+ rats. BrdU (green fluorescence) is incorporated in proliferating cells. BrdU-positive cells were evaluated in vehicle-treated Cy/+ rats (**A**) and everolimus-treated Cy/+ rats (**B**). The brush border of the proximal tubule is shown by a red fluorescence with an antibody against XT-2. More normal tubule structures and fewer BrdU-positive cells are visible in everolimus-treated Cy/+ rats compared to vehicle-treated Cy/+ rats. Bar scale: 100 μm.

Fig. 6. Everolimus retards loss of renal function in Cy/+ rats. BUN as a parameter of renal function was monitored on days 0, 17 and 36. The steady increase of BUN levels was reduced by 31% in Cy/+ rats after 5 weeks of everolimus treatment. ** $p < 0.001$ Cy/+ with vehicle versus Cy/+ with everolimus group. * $p < 0.05$ +/+ with everolimus versus +/+ with vehicle group.

show that everolimus treatment inhibited body weight and kidney weight gain in +/+ and Cy/+ rats. However, the effect on Cy/+ kidney weight was more pronounced than on +/+ kidney weight.

Everolimus Inhibited the Increase of Renal Cyst Size and the Number of Proliferating Epithelial Cells

Histological examination was performed on Han:SPRD rat kidneys by periodic acid-Schiff staining. Figure 4B shows that renal cysts predominantly exist in the cortex and to a lesser extent in the outer medulla. No cysts were found in the inner medulla in Cy/+ rats at 9 weeks of age. The everolimus treatment did not change the histology pattern in Cy/+ rats but it significantly reduced cyst density by 48% ($p < 0.001$) in Cy/+ rats (fig. 4C, D).

We then examined the effect of everolimus treatment on cyst epithelial cell proliferation by BrdU application in everolimus- or vehicle-treated Cy/+ rats (n = 3) on days 33, 34 and 35. Figure 5A demonstrates that BrdU predominantly labeled cyst epithelial cells in vehicle-treated Cy/+ rats. Everolimus strikingly reduced the number of BrdU-labeled TEC in the cortex (fig. 5B) and in the outer medulla (not shown) of Cy/+ rats.

Renal Functional Deterioration Was Stabilized in Everolimus-Treated Cy/+ Rats

The reduction of cyst growth and kidney weight by everolimus was accompanied by a significant amelioration of renal functional loss on day 36. The BUN level was 31% ($p < 0.001$) lower in Cy/+ rats compared with vehicle-

treated Cy/+ rats (fig. 6). Everolimus treatment did not completely prevent the renal functional deterioration in Cy/+ rats, as BUN levels in everolimus-treated Cy/+ rats were 28% ($p < 0.01$) higher than in vehicle-treated +/+ rats. Everolimus treatment also resulted in a 20% ($p < 0.05$) increase of BUN level in +/+ rats.

Discussion

There is current experimental evidence indicating that mTOR is out of control in inherited PKD. Aberrant signals through mTOR have been shown in different rodent models of PKD and in human ADPKD [4, 5]. Here we show that mTOR inhibition with everolimus efficiently retarded the development of renal cysts and ameliorated kidney function in Han:SPRD rats. These results extend previous studies with the mTOR inhibitor rapamycin, which also slowed the progression of PKD in the same rat model. Both mTOR inhibitors efficiently retarded disease progression, suggesting that the observed effects represent a class effect of mTOR inhibition.

Rapamycin and its analogue everolimus are both clinically used as immunosuppressants and approximately similar whole-blood drug levels are effective in preventing allograft rejection in rats and humans [16, 17]. In our previous study, relatively low rapamycin whole-blood levels (from 0.5 to 1.9 μg/l) led to reduced BUN values after 1 month (by –19%) and prevented cyst growth (by –18%) after 3 months of treatment. Compared to that, moderately higher everolimus whole-blood trough levels (between 5 and 7 μg/l) reduced BUN and cyst growth approximately 3-fold more than rapamycin, pointing to a profound dose effect of mTOR inhibition on cystic kidney disease progression. The bioavailability of oral rapamycin is low but increases markedly when given intraperitoneally. In the study of Tao et al. [5] the administration of rapamycin (0.2 mg/kg/day) intraperitoneally for 5 weeks in Han:SPRD rats reduced BUN (by –34%) and cyst volume density by (–40%). The rapamycin blood levels were not reported in their study, but the treatment was comparably effective in halting disease progression as everolimus treatment. Notably, Tao et al. also stated a significant reduction of body weight gain. In contrast, low trough level of rapamycin did not cause a significant body weight loss in our previous study.

Interestingly, treatment for 5 weeks with everolimus also caused a slight deterioration of renal function in normal +/+ rats, as shown by increasing BUN levels. It has been proposed that rapamycin or everolimus can promote glomerular lesions and impair TEC regeneration in certain conditions [18–20]. Like rapamycin, everolimus can also impair renal function [21]. In agreement with our study, it has been shown by others that 1 week of everolimus treatment by gavage feeding with a similar dosage (3 mg/kg/day) impaired renal function, in normal rats on a low-salt diet [21]. Furthermore, higher-dose (3 mg/kg/day) as opposed to a lower-dose (0.3 mg/kg/day) everolimus treatment worsened glomerular injury in a rat model of glomerulonephritis, suggesting again that everolimus could impair renal functions dose-dependently [22]. In our previous study the lower trough level of rapamycin did not increase BUN levels in +/+ rats. In future clinic studies using everolimus, the dosage needs to be adjusted carefully to balance the beneficial and adverse effects of everolimus in human ADPKD patients.

BrdU staining revealed that the number of proliferating renal epithelial cells was reduced by everolimus treatment. The same observation was made by Tao et al. [5] by using proliferating cell nuclear antigen staining in Han:SPRD rats. The effect of mTOR inhibitors on TEC is also demonstrated by the finding that rapamycin severely impairs the recovery from delayed graft function in patients who received a renal transplant and were treated with rapamycin [19, 20]. Rapamycin decreases the proliferation of TEC, and a hallmark of PKD is aberrant proliferation of renal epithelial cells [23]. Therefore, the major effect of mTOR inhibition in PKD could be the inhibition of renal tubular cell proliferation. However, we conducted this study in the early stages of PKD. In later stages of PKD, the expansion of growing renal cysts could occupy and squeeze the space for normal nephrons. Other pathophysiological events such as interstitial fibrosis, hypoxia and angiogenesis could then emerge. Therefore, it would be interesting to test if mTOR inhibitors could exert their beneficial effect beyond antiproliferative mechanisms in the later stages of PKD.

We conclude that the observed inhibitory effect on cyst progression with everolimus is a class effect of mTOR inhibitors. Everolimus could be a promising new drug to treat ADPKD patients.

Acknowledgments

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Chapter 3

Pulse mTOR inhibitor treatment effectively controls cyst growth but leads to severe parenchymal and glomerular hypertrophy in rat polycystic kidney disease

(Under review)

Pulse mTOR inhibitor treatment effectively controls cyst growth but leads to severe parenchymal and glomerular hypertrophy in rat polycystic kidney disease

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Short running title: everolimus withdrawal in rats with polycystic kidney disease

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Abstract

The efficacy of mammalian target of rapamycin (mTOR) inhibitors is currently tested in patients affected by autosomal dominant polycystic kidney disease. Treatment with mTOR inhibitors has been associated with numerous side effects. However, the renal specific effect of mTOR inhibitor treatment cessation in polycystic kidney disease is currently unknown. Therefore we compared pulse and continuous everolimus treatment in Han:SPRD rats. Four-week-old male heterozygous polycystic and wild-type rats were administered everolimus or vehicle by gavage feeding for 5 weeks, followed by 7 weeks without treatment, or continuously for 12 weeks. Cessation of everolimus did not result in the appearance of renal cysts up to 7 week post-withdrawal despite the re-emergence of the S6 kinase activity coupled with an overall increase in cell proliferation. Pulse everolimus treatment resulted in a striking non-cystic renal parenchyma enlargement and glomerular hypertrophy that was not associated with compromised kidney function. Both treatment regiments ameliorated kidney function, preserved glomerular-tubular connection and reduced proteinuria. Pulse treatment at an early age delays cyst development but leads to striking glomerular hypertrophy which might have a major impact on the treatment of patients with autosomal dominant polycystic kidney disease.

Key words: mammalian target of rapamycin (mTOR), everolimus, polycystic kidney disease

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease, resulting in progressive renal failure and end stage renal disease in adulthood [75]. The continuous growth of cysts is associated with increased tubular epithelial cell (TEC) proliferation leading to progressive cystic kidney enlargement and a loss of renal function [76].Currently specific treatments for human ADPKD other than supportive care do not exist.

The mammalian target of rapamycin (mTOR) is a key controller of cell growth and proliferation [77]. The pathway has two branches, which start with the mTOR complexes known as mTORC1, (mTOR, Raptor) and mTORC2 (mTOR, Rictor) [78]. The former is inhibited by sirolimus (rapamycin) and its analogue everolimus, while the later is not. The direct downstream targets of mTORC1, ribosomal protein S6 kinase (S6K) and 4E-BP1, in turn tightly regulate the downstream translational initiation machinery to control cell growth and proliferation [79].

The mTOR pathway is inactive in the healthy adult kidney. In this situation tuberin, an upstream inhibitor of mTOR, is attached to the cytoplasmic tail of the polycystin complex [45]. Mutations of the polycystin complex (which are the cause of ADPKD) direct tuberin to the cytoplasmic compartment resulting in a continuously activated mTOR pathway. The specific inhibition of this pathway with mTOR inhibitors can retard cyst growth and ameliorates kidney function loss in various murine models of PKD [45, 60-62, 80, 81]. Based on these promising pre-clinical studies, the efficacy of rapamycin and everolimus, immunosuppressants approved for kidney transplantation, are currently examined in clinical trials for ADPKD [82, 83]. Treatment

with mTOR inhibitors has been associated with numerous side effects, therefore short term or pulse treatment may offer an alternative treatment regimen to increase treatment tolerability. Some ADPKD patients currently enrolled in clinical trials testing efficacy of mTOR inhibitor treatment have been withdrawn from study drug due to adverse events. However, the renal specific effect of such an mTOR inhibitor withdrawal has so far never been examined. The purpose of the present investigation was therefore to study the effect of pulse compared to continuous everolimus treatment in Han:SPRD rats, a rodent model for polycystic kidney disease. Here we show for the first time, that the interruption of the mTOR treatment did not result in the appearance of renal cysts up to 7 weeks post-withdrawal. However, cessation of everolimus treatment resulted in striking glomerular and non-cystic renal hypertrophy, which was not associated with compromised kidney function. Pulse compared to continuous everolimus application differentially inhibited the mTOR pathway regulators AKT and GSK and the mTOR effectors rpS6 and 4E-BP1, pointing to a potential distinct biochemical role of mTORC1 and mTORC2 in the development and progression of PKD.

Materials and Methods

Animals

The Han:SPRD rat colony was established in our animal facility from a litter which was obtained from the Rat Resource & Research Center (Columbia, MO, USA). Heterozygous cystic (Cy/+) and wild type normal (+/+) rats were used in this study. Only male rats were used since cysts develop more rapidly in male compared with female rats [84]. The regulatory commission for animal studies, a local government agency, approved the study protocol. Rats had free access to tap water and standard rat diet.

Study drug

Everolimus microemulsion (20 mg/g) and vehicle microemulsion were kindly supplied by Novartis Pharma AG (Basel, Switzerland) and stored at -20°C. Everolimus and vehicle were diluted with tap water before oral administration.

Experimental protocol

Male Cy/+ and +/+ rats were weaned and then treated at 4 weeks of age with 3 mg/kg/day everolimus (Cy/+: n = 9; +/+: n = 5) or vehicle (Cy/+: n = 11; +/+: n = 8) (vehicle treatment [VT]) by gavage at a volume of 2 ml/kg/day for 12 weeks throughout (continuous treatment [CT]). In a different schedule everolimus was discontinued after 5 weeks, followed by 7 weeks without treatment (pulse treatment [PT]) (Cy/+: n = 5; +/+: n = 6). The dose of everolimus or vehicle was adjusted daily according to the body weight of the rats. All animals were sacrificed at week 16.

Blood chemistries

Tail blood was obtained from rats at week 4, 8, 12 and 16. Blood urea nitrogen (BUN) and creatinine were determined in plasma, whereas everolimus trough levels were measured in whole blood. All samples were stored at –20°C prior to measurement. BUN was analyzed by kinetic color testing and creatinine by the IDMS traceable modified Jaffé method. Everolimus blood levels were analyzed by HPLC-mass spectroscopy.

Determination of urinary protein excretion

24-hour urine samples were collected at week 16 in metabolic cages for selected rats ($n \geq 4$ for each group) and centrifuged. Urinary albumin was measured by a turbidimetric method (Roche Diagnostic, Basel Switzerland). Albuminuria was expressed as urinary albumin to creatinine ratios (mg/mmol). All measurements in blood and urine samples were performed by the Institute for Clinical Chemistry, University Hospital, Zurich, Switzerland.

Urine was also analyzed by non-reducing SDS-PAGE. Twenty µl urine samples were boiled for 5 minutes at 95°C and resolved in denaturing 7.5% and 15% polyacrylamide gels. Electrophoresis was followed by staining of the gels with Coomassie brilliant blue R250 for 30 minutes. The gels were destained and washed in double distilled water and dried for imaging.

Tissue processing

At the age of 16 weeks, all rats were sacrificed and kidneys were excised, decapsulated and weighed. For histological examination, one of the kidneys from each animal was sliced perpendicularly to the long axis at approximately 2 mm

intervals. Slices from the midportion of the kidneys were fixed in 4% buffered formalin and submitted to subsequent paraffin embedding. Up to 65 serial sections of 3 µm thickness per paraffin block were cut. Every second and third consecutive slide was stained with regular H&E (hematoxylin eosin) and PAS (periodic- acid Schiff) stain.

Morphometric measurements

The investigators of the morphometric measurements were blinded to experimental groups and repeated measurements were performed without knowledge of each other's results.

Determination of cyst volume density

Cyst volume density (CVD) was assessed on Periodic acid-Schiff (PAS) stained sections by morphometry, using the method of point-counting [85]. 4-6 micrographs were obtained for one section per rat for morphometry as described before [62]. The pixel size of the micrographs was 1300 × 1030 (1.34 µm/pixel). An orthogonal grid with a line spacing of 150 pixels was used.

Glomerular volume measurement

Mean glomerular volume (GV) was determined from the mean glomerular capillary tuft area (A_G) by light microscopy of PAS sections. The areas were determined by light microscopy and analyzed by a dedicated software (Analysis 3.0, Soft Imaging System, Münster, Germany) as the average area of 50 glomerular profiles (the capillary tuft omitting the proximal tubular tissue and Bowman's capsule) for each animal. GV was calculated using the formula: $GV = ß/k \times (A_G)^{3/2}$ where ß = 1.38, which is the shape coefficient for spheres (the idealized shape of glomeruli), and *k* =

1.1, which is a size distribution coefficient, and A_G, which is the glomerular capillary tuft area.

Assessment of glomerular-tubular connection

Serial sections of everolimus treated and non-treated heterozygous polycystic (Cy/+) and wild-type normal (+/+) rat kidneys of 2 µm thickness were cut and stained with Periodic acid-Schiff (PAS). On the sections glomeruli were counted and each glomerulus examined for the presence of a urinary pole. This procedure was continued until 50 urinary poles were detected. If a section did not contain 50 urinary poles, another section of that kidney was evaluated. This section was at least 50 µm from the previous one to ensure that the same glomerulus was not evaluated twice. Sections were independently evaluated by two investigators and the results averaged. The number of glomeruli to obtain 50 urinary poles was used as a measure of glomerular-tubular connection, i.e. the higher the numbers of counted glomeruli the more atubular glomeruli exist in a kidney.

Antibodies for immunostaining and Western blotting

The following antibodies were used: rabbit anti-human polyclonal antibody recognizing Ki-67 antigen (clone MIB 1; Dako Diagnostic, Zug, Switzerland), rabbit anti-mTOR (7C10), mouse anti-Phospho-Akt (Ser473), rabbit anti-Phospho-GSK-3α/β (Ser21/9), rabbit anti-Phospho-4E-BP1 (Thr37/46), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236), rabbit anti-Phospho-S6 Ribosomal Protein (Ser240/244) (Cell signaling Technology, Beverly, MA, USA), rabbit anti-actin (Sigma, St. Louis, MO, USA), Rabbit anti-RPS6 Antibody (Bethyl Laboratories, Montgomery, TX, USA), rabbit anti-Akt1/2/3 (H-136) (Santa Cruz Biotechnology,

Santa Cruz, CA, USA). Goat anti-rabbit HRP-conjugated antibody (Dunn Labortechnik GmbH, Asbach, Germany) and sheep anti-mouse HRP-conjugated antibody (GE Healthcare, Buckinghamshire, UK) were used as the secondary antibodies.

Immunohistochemical detection of proliferation

Immunhistochemistry was performed on paraffin sections of formalin-fixed tissues using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA). For antigen retrieval, the slides were heated with Ventana cell conditioner one (mild protocol). Anti-Ki-67 (dilution 1:20) antibody was detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. Slides were counterstained with hematoxylin. Micrographs were made randomly using the x200 magnification of the light microscope. The proximal tubule was identified by the brush border, detectable in background fluorescence and was controlled with PAS stain on a corresponding serial section. Nuclei of proximal epithelial cells positive for Ki-67 were counted on 100 consecutive tubules. Tubule with less than 5 cells was not included for assessment. Proliferation index of TECs was calculated as the percentage of positively stained Ki-67 cells to total cells. The staining was scored independently by two observers.

Protein extraction and Western blotting analysis

Snap frozen kidney tissue was homogenized by the automated homogenizer (Precellys 24; Stretton Scientific, Stretton, UK) in freshly made tissue protein extraction reagent (T-PER, Pierce Bioscience, Rockford, IL, USA) containing 1 mM PMSF, 0.5 M EDTA, Halt Protease Inhibitor cocktail and Halt Phosphatase Inhibitor.

Homogenates were centrifuged twice at 4°C at 13,000 g for 15 min, and supernatants were stored at -80°C.

60 µg protein samples and a reducing loading buffer were mixed and boiled for 5 min at 95°C. Samples were loaded on a 7.5% SDS-PAGE gel. After migration, the proteins of the gels were transferred onto a 0.2 µm PVDF membrane (BioRad, Hercules, CA, USA). The blots were blocked by drying for 15-30 min at room temperature and were then incubated overnight at 4°C in a 5% BSA/PBST buffer containing primary antibodies against actin (dilution 1:20,000), mTOR (1:1000), RPS6 (1:5000), p240/244 S6 (1:3000), p235/236 S6 (1:1000), Akt1/2/3 (1:1000), p21/9 GSK-3α/β (1:1000) or p37/464E-BP1 (1:1000). Secondary antibodies against rabbit (1:10,000) or mouse (1:10,000) were applied for 45 minutes at room temperature after 3 times wash with PBST. After 4 times wash with PBST, the membranes were incubated for 5 min in chemiluminescence substrate kit (ChemiGlow; Alpha Innotech, San Leandro, CA, USA). Blots were visualized with Chemi-Doc XRS system (BioRad. Richmond, CA, USA). The molecular weight of the bands of interest was determined by a pre-stained protein standard (SeeBlue Plus 2 Prestained Standard, Invitrogen, Carlsbad, CA, USA). Quantification of protein expression was normalized to actin using densitometer and displayed as percentage changes over vehicle treated Cy/+.

Statistical analyses

Statistical analyses were performed by unpaired t-test or one-way ANOVA with the Newman-Keul's post-hoc test using GraphPad Prism version 5.0 (Graph Pad Inc.,

San Diego, CA, USA). All data are expressed as means ± SEM and $P < 0.05$ was considered as statistically significant.

Results

In this study, everolimus was applied daily by gavage to 4 weeks old male Cy/+ and +/+ rats for 12 weeks (continuous treatment [CT]) or in a different schedule everolimus was discontinued after 5 weeks of treatment, followed by 7 weeks without treatment (pulse treatment [PT]). The everolimus whole blood levels were similar in both treatment groups at week 9 (Table 1). Judging by physical appearance, the treatment was well tolerated. None of the everolimus treated rats died, and all control rats survived. Figure 1 shows that body weight increased steadily in untreated rats suggesting that the cystic kidney disease by itself did not influence the normal growth of the animals. Everolimus treatment had an inhibitory effect on body weight gain and the withdrawal of everolimus at week 9 resulted in a catch-up of body weight until week 16.

Continuous as well as pulse everolimus treatment result in cyst reduction and renal function preservation

The two treatment schedules, pulse and continuous everolimus application, efficiently reduced cyst grow and ameliorated the kidney function in Cy/+ compared to vehicle treated Cy/+ (Figure 1 and 2). Pulse treatment reduced cyst volume density (-76%, $P<0.01$), lowered BUN (-47%, $P<0.001$) and serum creatinine levels (-47%, $P<0.001$). Likewise cyst growth (-54%, <0.01), BUN (-49%, $P<0.001$) and serum creatinine levels (-40%, $P<0.001$) were reduced in the everolimus continuous group.

Kidney weight does not differ between pulse and vehicle treated animals due to significant parenchymal and glomerular hypertrophy in pulse treated animals

Polycystic kidney disease progression in Cy/+ led to an approximately two-fold increase of kidney weight and size compared to wild type rat (Figure 2A and 2B). Everolimus continuous treatment partly reduced this enlargement as shown by -32% ($P< 0.001$ vs. vehicle) reduced 2-kidney weight to total body weight (2K/TBW) ratio. In contrast, pulse and vehicle-treated Cy/+ rats had equal 2K/TBW ratios at week 16. This apparent absence of a kidney weight reducing effect despite retardation of cyst growth by pulse everolimus treatment was unexpected. We therefore assessed the proliferation of TEC by the quantification of Ki67 positive cells. Figure 4A shows representative kidney sections of all experimental groups, revealing that the approximately 7-fold higher proliferation rate of TECs in vehicle-treated Cy/+ compared to +/+ rats. mTOR inhibitors have besides its immunosuppressive effect a well known anti-proliferative action [86]. Indeed, continuous and to a lower extent pulse everolimus treatment reduced the augmented proliferation of TEC and cystogensis in Cy/+ rats. A further histomorphological analysis revealed that the glomerular volume was markedly increased in pulse treated Cy/+ (77%, $p < 0.01$ vs. vehicle and continuous everolimus-treated Cy/+) (Figure 2A and 2D). A nephron deficit or the impairment of the glomerular-tubular connection (GTC) leads to compensatory enlargement of remaining glomeruli. We therefore estimated the number of nephrons by counting glomeruli, as a surrogate measure, and found similar values in all groups (data not shown). The integrity of the GTC was assessed by counting the number of glomeruli to identify 50 urinary poles. This number, an indicator of glomerular-tubular disconnection, was three-fold increased in vehicle-treated Cy/+ rats compared to +/+ indicating that polycystic kidneys contain large numbers of atubular glomeruli (Figure 2E). Both everolimus regimens reduced this number in Cy/+ to the level of +/+ rats

demonstrating the preservation of the glomerular-tubular integrity by mTOR inhibitor treatment.

Pulse and continuous everolimus treatment attenuated proteinuria in Cy/+

The enlargement of glomeruli, the development of polycystic kidney disease and treatment with mTOR inhibitors have been associated with increased urinary protein excretion [87-89]. We therefore measured proteinuria, albuminuria and performed urine SDS-PAGE of 24h urine collection (Figure 3). Vehicle treated Cy/+ had approximately four-fold higher urinary total protein and albumin excretion compared to +/+ (Figure 3B). Both everolimus treatment schedules reduced markedly proteinuria. Continuous everolimus treatment reduced the urinary total protein to creatinine and albumin to creatinine ratio by 79% ($P<0.001$ vs. vehicle-treated Cy/+) and by 77% ($p < 0.001$), respectively. Pulse treatment was similar efficient and reduced the ratios by 48% ($P<0.001$) and 44% ($p <0.01$), respectively. Urine SDS-PAGE revealed a strong band for albumin in samples of vehicle-treated Cy/+, a band that was reduced by both treatment regimens (Figure 3A). When comparing urine samples of Cy/+ vs. +/+ no additional differences in the band pattern were found by urinary SDS-PAGE (Figure 3C). These results indicate that mTOR inhibitor treatment reduced the augmented urinary protein excretion associated with polycystic kidney disease. This beneficial effect was more pronounced when everolimus was given continuously.

Differently effect of pulse and continuous everolimus treatment regimens on mTOR pathway

Previously published *in vivo* studies and the enhanced understanding of polycystin complex disruption associated delocalization of tuberin supports the hypothesis that mTOR is activated in polycystic kidney disease [45]. However, to the best of our knowledge, the expression levels of these kinases have not been previously assessed after mTOR treatment withdrawal. Before investigating the effect of everolimus treatment, we evaluated the activation of mTOR-regulated downstream targets (p4E-BP1 and pS6) as well as upstream regulators (pAkt) in Cy/+ kidneys. Phosphorylation of S6 (ser235/26 and ser240/244) and 4E-BP1, two readouts of mTORC1 signaling as well as p473 Akt and it`s downstream target glycogen synthase kinase-3 (GSK3), the readout of mTORC2 activity, were examined by western blot. Figure 5 shows a significant up-regulation of phosphorylated S6, 4E-BP1 as well as Akt and GSK3 in vehicle-treated Cy/+ compared to +/+ rats, demonstrating constitutive activation of mTORC1 and mTORC2 in polycystic kidneys.

Next, we evaluated the effect of continuous long-term mTOR inhibitor treatment on the mTOR complexes. Compared to vehicle treated Cy/+ rats, continuous everolimus treatment blocked phosphorylation of S6 whereas p4E-BP1, pAkt and pGSK3 remained unchanged (Figure 5). These results suggest that continuous everolimus treatment only partly inhibited mTORC1 and that the over activated mTORC2 was insensitive to the mTOR inhibitor. Our findings further support a recent *in vitro* study describing such a differentially phosphorylation of S6 and 4E-BP1 after long-term mTOR inhibitor treatment [90].The functional consequences of a differential inhibition of the mTORC1 effectors along with an over active mTORC2 are not well known. In our PKD rat model, the continuous everolimus-mediated partial mTORC1 blockade reduced efficiently TEC proliferation and cyst growth without suppressing the over

activation of AKT/mTORC2 suggesting that mTORC2 plays a minor role in cyst growth.

Everolimus has a short half life of approximately 12 hours and even after oral doses ≥20 mg/wk, S6 kinase activity in peripheral-blood mononuclear cells was inhibited not longer than 10 days in cancer patients [91]. We therefore hypothesized, that mTOR activity will recover after everolimus withdrawal for up to 7 weeks accompanied by a re-growth of cysts. Strikingly, everolimus withdrawal restored only partly the activity of mTORC1 whereas the signaling through Akt/mTORC2 pathway was shut down (Figure 5). These results indicate that recovering of kidney weight and glomerular enlargement in pulse treated Cy/+ can not be attributed to upregulated mTORC2 activity and that partial active mTORC1 enabled parenchymal hypertrophy. In summary, components of the mTORC1 and mTORC2 are differently expressed upon mTOR inhibitor treatment suggesting so far unknown regulators.

Discussion

The present study shows that removal of everolimus did not result in the appearance of renal cysts up to 7 weeks post-withdrawal despite the reactivation of the mTOR pathway coupled with an overall increase in cell proliferation. Cessation of everolimus treatment resulted in a non-cystic renal parenchymal and glomerular hypertrophy, which was not associated with compromised kidney function.

It is generally accepted that everolimus is a global inhibitor of translation by blocking signaling through mTORC1 and it might be hypothesized that relief of mTOR inhibition would result in reactivation of the pathway and subsequent growth and proliferation of renal cysts. In our study it appears that prolonged everolimus treatment restored mTOR activity by rendering mTORC1 to be mTOR inhibitor resistant towards 4E-BP1 and after withdrawal towards S6 in Cy/+ kidneys. Such a differential regulation of mTORC1 compounds upon mTOR inhibitor application has been recently shown *in vitro* but so far not *in vivo*. In this *in vitro* long-term mTOR inhibitor study, p4E-BP1 remained active and mediated cap-dependent translation although the biological consequences of partial mTORC1 activity remains unexplored. In our study this partial mTORC1 activity did not mediate cyst growth and consequently the impact of mTORC1, in particular p4E-BP1 on cyst growth seems to be at least limited.

Continuous mTOR inhibitor treatment markedly reduced cell proliferation and cyst growth whereas the aberrant activated p473 Akt/mTORC2 pathway, which plays an important role in cell survival and tumor growth, was not affected. Recent studies have shown that mTOR inhibitors have the ability to inhibit mTORC2 assembly and

Akt activity. However the sensitivity of mTORC2 to mTOR inhibitors is time and dose dependent and is further modified by the level of phosphatidic acid (PA), factors that may have rendered mTORC2 to become mTOR inihitor resistant in everolimus treated Cy/+ [78, 92]. Second, rapamycin induces feedback activation of the mTOR upstream pathway most likely via S6 kinase and disrupted feedback loops can render cells to become mTOR inhibitor resistant [93-95]. The inverse correlation of S6K activity and Akt/GSK3 in pulse and continuous everolimus treated Cy/+, suggests an intact negative feedback loop in kidneys of Han:SPRD rats.

Our finding of a non-cystic renal hypertrophy and re-activation of the ribosomal protein S6 kinase which was not associated with further improved kidney function supports the hypothesis of a futile repair program, as proposed by Weimbs et al. Thus, the observed increase in cell proliferation after everolimus withdrawal may represent an early event preceding cyst formation [37, 96]. Taken together, our findings confirm and extend the knowledge on the role of the mTOR pathway in cyst growth and suggest that at least partial mTOR/S6K activity is needed for cyst growth.

Effect of everolimus treatment on glomeruli

It is a characteristic of ADPKD that despite continuous cyst growth the glomerular filtration is maintained for decades due to hyperfiltration. This compensatory mechanism tends to increase the size of glomeruli in human ADPKD [27]. In our study glomerular volume was similar in +/+ and continuous everolimus treated Cy/+. However after everolimus withdrawal, glomerular volume increased suggesting that the discontinuation of the mTOR inhibitor permitted the development of compensator hypertrophy. Indeed, the mTOR signaling pathway plays an important role in

glomerular hypertrophy as in several disease models activation of the mTOR pathway leads to glomerular hypertrophy and mTOR inhibitors prevented or retarded glomerular hypertrophy [97-99].

Notably, this hypertrophic glomeruli were almost always connected to open proximal tubules pointing to the importance of an intact glomerular-tubular connection for adaptive processes in the glomerulus [100]. The glomerular-tubular junction is vulnerable to toxins and stress like tubular obstruction, inflammation, ischemia and proteinuria, conditions that occur already early in the course of PKD [87, 101]. Continuous and pulse everolimus treatment prevented the Cy/+ associated glomerular-tubular disconnection with equal efficacy probably due to the reduction of cyst growth and urinary protein excretion. Taken together, both everolimus treatment regimen preserved the glomerular-tubular connection intact whereas the discontinuation of everolimus permitted the development of compensatory glomerular hypertrophy.

Our study has several limitations. First, mTOR inhibitors reduce cell size and we cannot exclude that increasing cell size upon withdrawal of everolimus contributed to the kidney growth [102, 103]. Second, the Han:SPRD rat is an imperfect model for human ADPKD. In the Han:SPRD rat the mutated gene encodes for samcystin and cysts are mostly of proximal origin whereas in human ADPKD the genes *PKD1* and *PKD2* are mutated and cysts origin from all parts of the nephron. Although, the slow disease progression and the gender dysmorphism resemble ADPKD phenotypically, the relevance of our findings for ADPKD has to be interpreted in the context of the animal model. Third, cyst development peaks at 8 – 10 weeks in Han:SPRD rats

which is followed by a development of interstitial fibrosis and decrease in renal size. Therefore it is possible that cyst development was equally inhibited during weeks 4 – 9 by both treatment regimens but the discontinuation of everolimus permitted compensatory mechanisms. Finally we observed a significant loss of body weight in everolimus treated rats, which was also found in several previous similar studies [60, 80, 81]. In the absence of pair fed control groups, we cannot exclude that the body weight change influenced mTOR-mediated cyst growth. However, in a previous study we showed that a low concentration of rapamycin significantly inhibit cyst growth and mTOR activity independently of body weight changes [62].

In conclusion, our study shows for the first time that everolimus withdrawal leads to a renal, non-cystic hypertrophy without the reemergence of renal cysts. In the past, animal studies pointed to a prominent role of the mTOR pathway in the cyst growth and progression of polycystic kidney disease and prompted the initiation of several clinical studies to examine the effectiveness of mTOR inhibitors in patients affected by ADPKD. Our data suggest a potential distinct biological role of the mTORC1 and mTORC2 pathway in cyst growth. The renal specific effect of everolimus, in particular after everolimus withdrawal are of considerable clinical relevance in the light of ongoing clinical trials testing efficacy of mTOR inhibitors in ADPKD patients.

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Figures legends

Figure 1

Body weight gain (A, B) of +/+ (open symbols) and Cy/+ (closed symbols) rats with vehicle (VT = circle), continuous everolimus (CT = square) and pulse everolimus (PT = triangle) treatment from week 4 to 16. ***p < 0.001. BUN (C, D) and serum creatinine (E, F) levels were measured in +/+ (open symbols) and Cy/+ (closed symbols) rats with vehicle treatment (VT= circle), continuous everolimus treatment (CT = square) and pulse everolimus treatment (PT = triangle) from week 4 to 16. **p < 0.01, ***p < 0.001.

Figure 2

Kidney histology was evaluated on PAS sections in +/+ (A-C) and Cy/+ (D-E) rats. Magnifications were x5 for the whole kidney images and x230 for the inserts. Representative images are shown. (G) 2-kidney to total body weight ratios (2K/TBWt) were measured in +/+ (open bars) and Cy/+ (filled bars) rats at week 16. (H) Cyst volume densities (CVD) were measured for +/+ (open bars) and Cy/+ (filled bars) rats. (I) Mean glomerular volume (GV) were measured for +/+ (open bars) and Cy/+ (filled bars) rats. (J) GT disconnection was assessed by counting glomerular number till 50 glomeruli with urinary pole were identified. **P < 0.01, ***p < 0.001, ns: not significant.

Figure 3

(A) Proteinuria in 24-hr urine was analyzed by 7.5% SDS-PAGE (B) and quantified by calculating the urinary albumin to creatinine ratios in +/+ (open bars) and Cy/+

(filled bars) rats. (C) Low molecular weight urinary protein was analyzed by 15% SDS-PAGE. **P < 0.01, ***p < 0.001.

Figure 4

Ki67 staining on +/+ and Cy/+ kidney rats. (A) Representative images are shown Magnifications were x200. (B) Tubular proliferation index in +/+ (open bars) and Cy/+ (filled bars) rats. ***p < 0.001. Representative images are shown.

Figure 5

(A) Western blot analysis of, pSer240/244 S6, pSer235/236 S6, total S6 protein, pThr37/46 4E-BP1 and total mTOR are shown. (B) Western blot analysis of pSer473 Akt, total Akt and pSer21/9 GSK3 α/β are shown. Actin served as internal control. Quantification of protein expression was normalized to actin using densitometer and displayed as percentage changes over vehicle treated Cy/+ (mean values of VT Cy/+ were set to 1). Representative data were shown from at least three independent experiments. *P < 0.05, **P < 0.01, ***p < 0.001.

Table

Table 1. Everolimus whole blood trough levels.

Everolimus whole blood trough levels (µg/l) in +/+ and Cy/+ at week 9 for continuous and pulse treatment and at week 16 for continuous treatment regimen. The everolimus whole blood levels were similar in the continuous (P=0.4) and pulse (P=0.7) treated animals at week 9. Mean (SEM).

	+/+		Cy/+	
	Continuous	Pulse	Continuous	Pulse
week 9	4.7 ± 0.4	5.8 ± 0.4	5.8 ± 0.4	6.7 ± 0.9
week 16	4.5 ± 0.3	_	6.2 ± 0.7	_

Figures

Figure 1

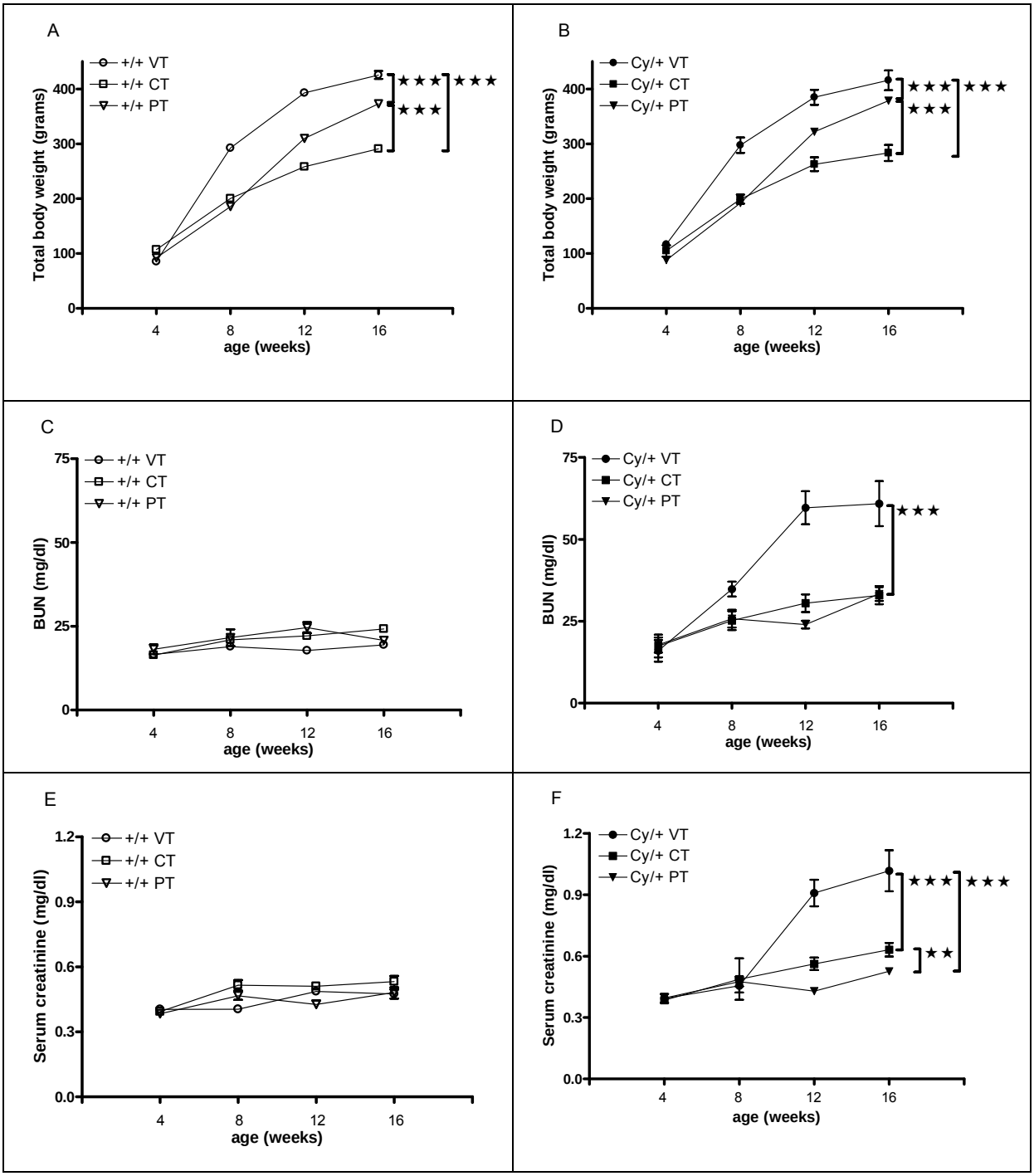


Figure 2

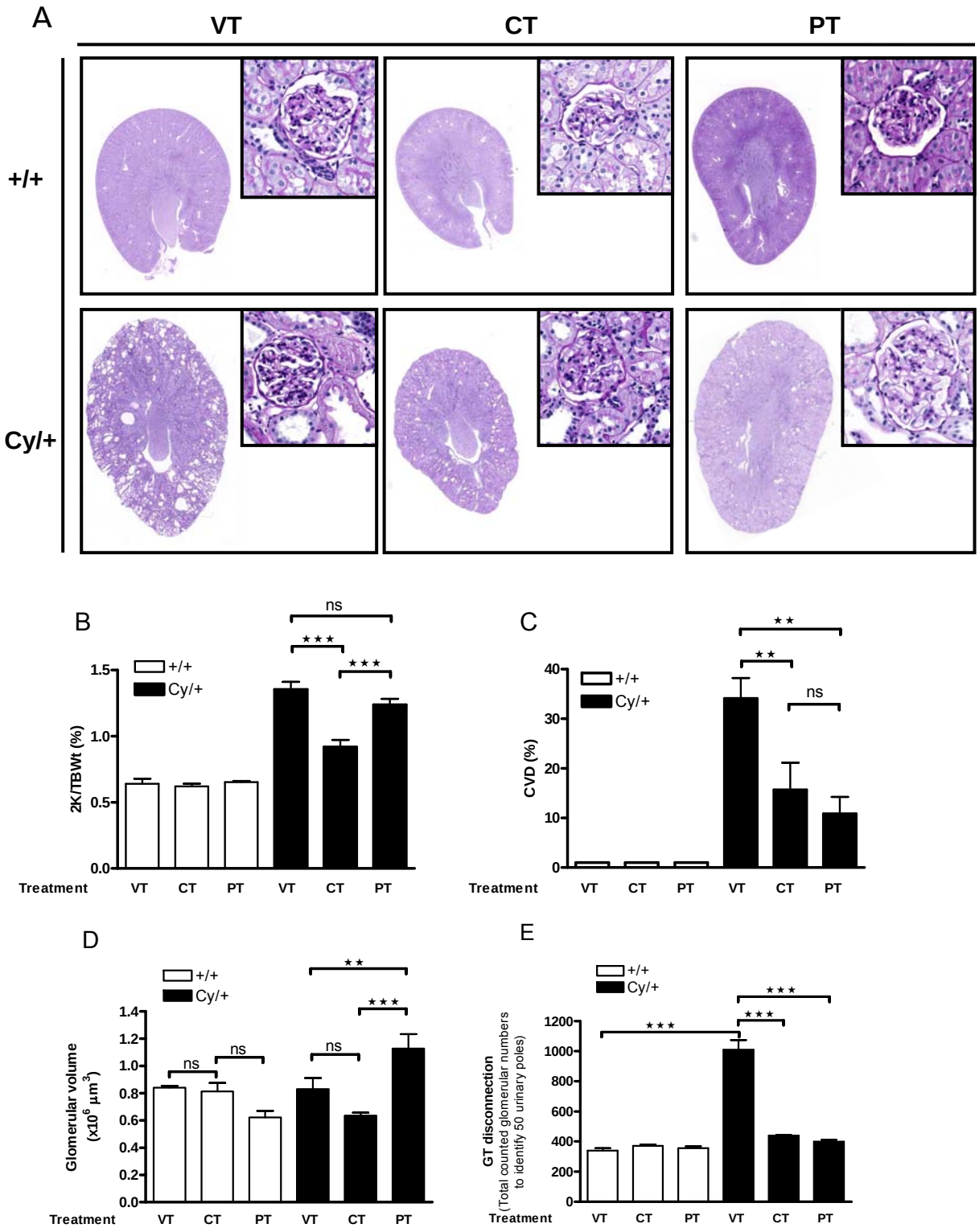

A
VT
CT
PT
+/+
Cy/+
B
2K/TBWt (%)
+/+
Cy/+
ns
Treatment
VT CT PT VT CT PT
C
CVD (%)
ns
D
Glomerular volume (x10^6 µm^3)
E
GT disconnection
(Total counted glomerular numbers to identify 50 urinary poles)

Figure 3

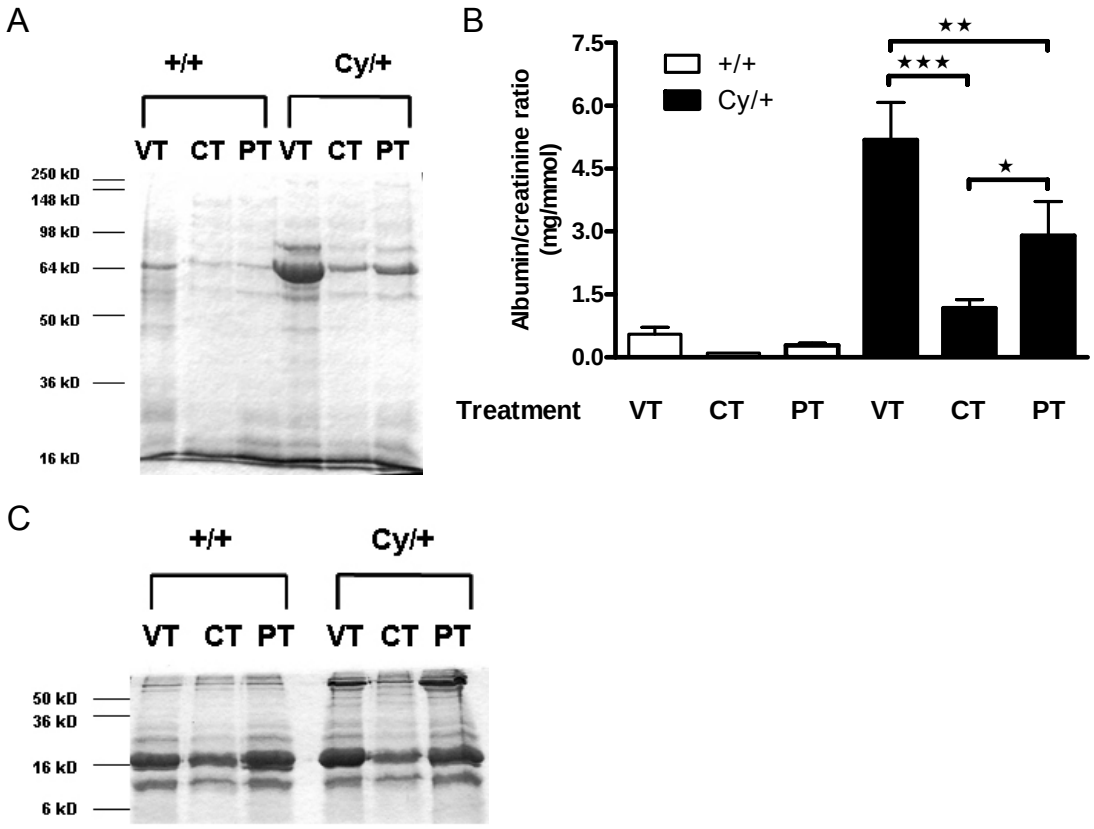

A
+/+
Cy/+
VT CT PT VT CT PT
250 kD
148 kD
98 kD
64 kD
50 kD
36 kD
16 kD
B
Albumin/creatinine ratio (mg/mmol)
7.5
6.0
4.5
3.0
1.5
0.0
+/+
Cy/+
★★
★★★
★
Treatment VT CT PT VT CT PT
C
+/+
Cy/+
VT CT PT VT CT PT
50 kD
36 kD
16 kD
6 kD

Figure 4

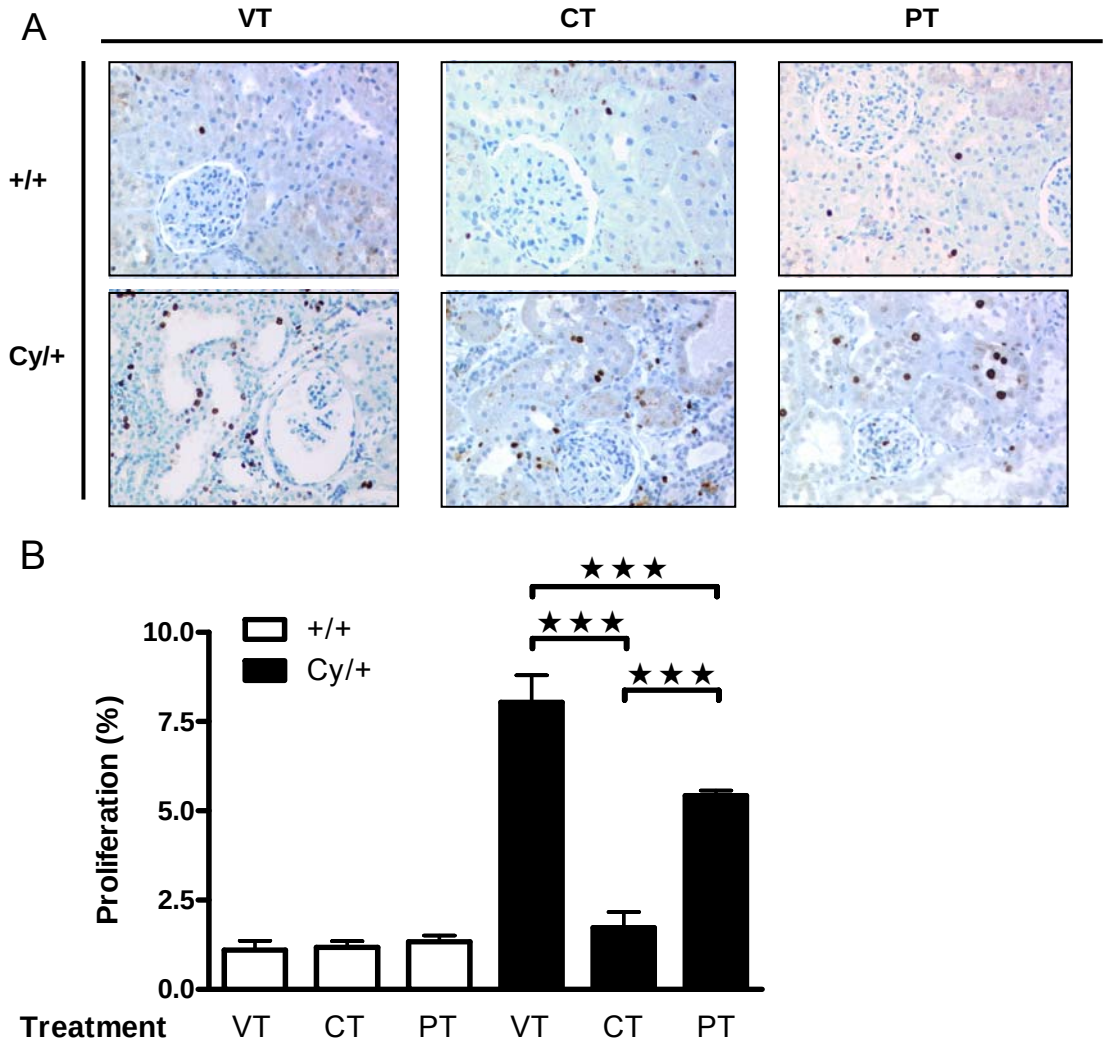

A
VT
CT
PT
+/+
Cy/+
B
★★★
★★★
★★★
10.0
7.5
5.0
2.5
0.0
+/+
Cy/+
Proliferation (%)
Treatment
VT
CT
PT
VT
CT
PT

Figure 5

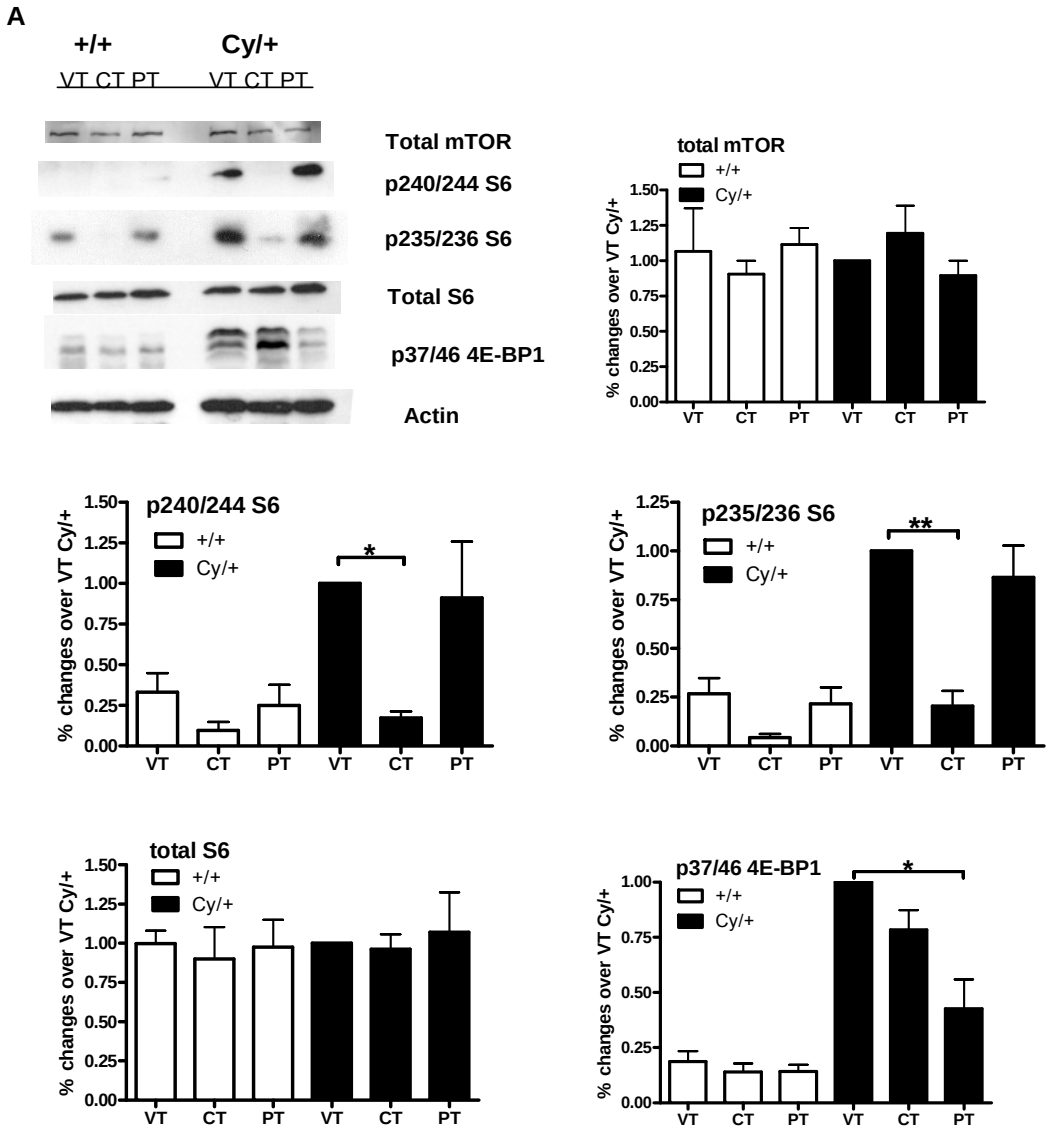
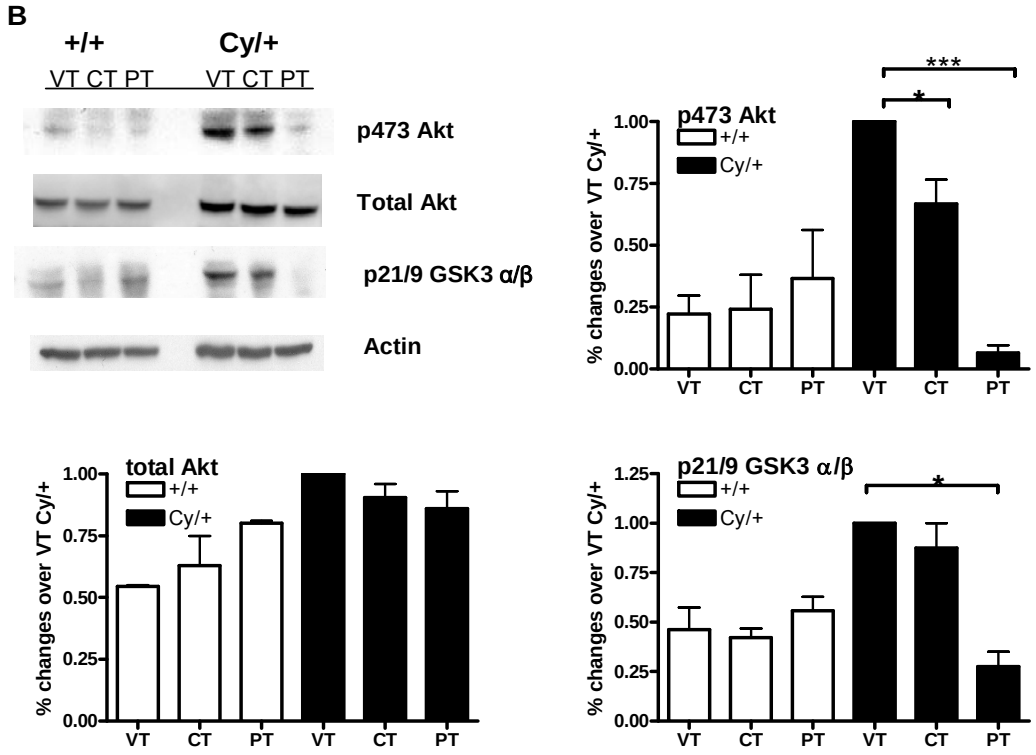

A
+/+
Cy/+
VT CT PT
VT CT PT
Total mTOR
p240/244 S6
p235/236 S6
Total S6
p37/46 4E-BP1
Actin
total mTOR
p240/244 S6
p235/236 S6
total S6
p37/46 4E-BP1
% changes over VT Cy/+
+/+
Cy/+
VT
CT
PT
*
**

Figure 5


B
+/+
Cy/+
VT CT PT VT CT PT
p473 Akt
Total Akt
p21/9 GSK3 α/β
Actin
p473 Akt
+/+
Cy/+
% changes over VT Cy/+
1.00
0.75
0.50
0.25
0.00
***
*
VT CT PT VT CT PT
total Akt
+/+
Cy/+
% changes over VT Cy/+
1.00
0.75
0.50
0.25
0.00
VT CT PT VT CT PT
p21/9 GSK3 α/β
+/+
Cy/+
% changes over VT Cy/+
1.25
1.00
0.75
0.50
0.25
0.00
*
VT CT PT VT CT PT

Chapter 4

Low dose of everolimus inhibits mTOR-mediated proliferation and cyst aggregation without inducing apoptosis in Cy/+ renal tubular epithelial cells

Low dose of everolimus inhibits mTOR-mediated proliferation and cyst aggregation without inducing apoptosis in Cy/+ renal tubular epithelial cells

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Abstract:

Everolimus is currently clinically tested as treatment for autosomal dominant polycystic kidney disease (ADPKD) and the mechanism of action is incompletely explored. Previous data has shown that everolimus cause dose-dependently adverse events that may constrain a potential use in ADPKD. Thus, we investigated the effects and mechanism of a wide range of everolimus levels on tubular epithelial cells derived from Han:SPRD rats, an animal model for polycystic kidney disease. Primary 2D and 3D cultures of tubular epithelial cells from wild type (+/+) and Cy/+ Han:SPRD rats were cultured in medium containing serum with everolimus levels from 0.01 nM to 100 nM. The population doubling time was 15.5±0.6h for Cy/+ and 21.9±4.1h for +/+ TECs (p=0.012). 0.1 nM everolimus reduced cell number and DNA synthesis with no effect on apoptosis. Phosphorylation of mTOR, S6 kinase and S6 in Cy/+ were dose-dependently reduced by everolimus starting from 0.1 nM in parallel with effect on cell number and DNA synthesis. The aggregate diameter of Cy/+ was higher compared to +/+ and everolimus selectively reduced the Cy/+ aggregate size. These data show that an everolimus level of 0.1 nM effects cell proliferation, aggregate size and phosphorylation of mTOR pathway effectors.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent hereditary kidney disease with a prevalence between 1:400 and 1:1000 and accounts for 7-10% of all patients requiring renal replacement therapy [75]. The relentless development and growth of innumerable cysts lead to progressive destruction of the normal renal parenchyma and massive enlargement of the kidneys [62, 63, 104]. Currently there is no specific treatment for ADPKD other than supportive care, and dialytic treatment or renal transplantation when patients have reached end stage renal disease.

Several molecular mechanisms contributing to cyst formation have been proposed, including altered intracellular signaling leading to a dysbalance in epithelial cell proliferation and apoptosis. The mTOR (mammalian target of rapamycin), an atypical serine/theronine kinase plays a central role in modulating cellular proliferation, and apoptosis [105]. This pathway is aberrantly activated in polycystic kidneys of different rodent models for ADPKD, as well as in cyst-lining cells in human ADPKD [45, 62, 81]. Everolimus (Certican®) and sirolimus (Rapamune®) are two specific and potent mTOR inhibitors that efficiently slow disease progression in the Cy/+ rat, a rodents models for polycystic kidney disease (PKD). Thus, key features of PKD are tubular cell proliferation, suggesting that mTOR inhibitors act via its anti-proliferative, and pro-apoptotic action.

Everolimus and sirolimus are currently clinically tested as treatment for ADPKD patients [NCT00346918 and NCT00414440]. However, mTOR inhibitors cause dose-dependently adverse events that may constrain a potential long-term use in ADPKD.

Dose-finding studies in ADPKD patients are planed but will last years and involve a large number of patients as the disease progresses slowly. Because of these potential detrimental effects, we aimed to determined the renal tubular epithelial cell-drug concentration relationship for everolimus *in vitro* to define the lowest inhibitory dose of everolimus of Cy/+ tubular epithelial cells proliferation.

We studied the effect of everolimus in primary 2D and 3D cultures of renal tubular epithelial cells from male Cy/+ rat and provide a number of novel findings. The augmented cell proliferation was inhibited by a remarkable low dose of everolimus in parallel with a dose-dependent specific inhibition of mTOR pathway with no effect on apoptosis. The aggregate diameter of Cy/+ was higher compared to +/+ and everolimus selectively reduced the Cy/+ aggregate size.

Materials and methods

Renal tubular epithelial cell isolation

The Han:SPRD rat colony was established in our animal facility from a litter which was obtained from the Rat Resource & Research Center (Columbia, MO, USA) and kept under local regulation and guidelines. Heterozygous cystic (Cy/+) and wild-type normal (+/+) male rats, aged 2-4 months were used in this study. Primary renal epithelial cells were isolated as follows. Kidneys were minced and digested by 1 mg/ml collagenase with gentle agitation for 1 hour at 37°C. Suspension was allowed to sediment 1 minute twice. Cells were collected by harvesting the supernatant and then washed 3 times with 10% FBS/HBSS.

Monolayer cell culture

Isolated cells were re-suspended in K1 medium (1:1 mixture of Dulbecco's modified Eagle's medium (Invitrogen) and Ham's F-12 medium (Invitrogen) supplemented with 5% FBS (Invitrogen), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES; Invitrogen) (pH 7.4), 42 mM sodium bicarbonate (Sigma), 5 g/ml insulin (Sigma), 50 nM hydrocortisone (Sigma), 5 g/ml transferring (Sigma), 5 pM triiodothyronine (Sigma), 100 IU/ml penicillin (Invitrogen), and 100 g/ml streptomycin (Invitrogen)) and incubate at 37°C, 95% O_2, 5% CO_2 on 10 μg/ml collagen (Sigma) coated 100 mm Falcon plastic petri dishes or 96-well plates for indicated experiments. The percentage of successful plated TEC was different for Cy/+ and +/+ (13% vs. 1.1%). Thus to reach the same degree of cell confluence at day 1, isolated TECs from +/+ or Cy/+ rats were seeded at 5-10 x 10^6 or 5-10 x 10^5 cells/ml, respectively. At following day (day 1), TECs reached 10-20% confluence and were washed 2 times with HBSS and replaced with fresh K1 medium in the absence or in the presence of

20 ng/ml rat epidermal growth factor (EGF; R&D). Positive staining for epithelial marker cytokeratin and negative staining for CD31 were obtained to characterize tubular epithelial cells (TECs).

Study drug

Everolimus as dry powder was kindly supplied by Novartis (Basel, Switzerland) for *in vitro* study. The powder was dissolved in ethanol and stored as 10 mmol/L stock solution at – 20°C.

Total protein assay

TECs from +/+ or Cy/+ rats were cultured in 100 mm Falcon plastic petri dishes in the presence of various concentrations (0.01nM to 100nM) of everolimus, rat epidermal growth hormone (EGF), minimal medium (K1 medium supplemented with 0.02% FBS, in the absence of growth hormones) or in normal K1 medium from day 1 for indicated experiments. Cell lysates were prepared by scraping cells from dishes using protein extraction reagent (T-PER, Pierce Bioscience, Rockford, IL, USA) containing 1 mM PMSF, 0.5 M EDTA, Halt Protease Inhibitor cocktail and Halt Phosphatase Inhibitor). Total protein content of lysates was measured using BCA assay (Pierce Bioscience, Rockford, IL, USA) according to the manufacturer's instructions.

Colorimetric cell number assay

TECs from +/+ or Cy/+ rats were cultured in 96-well plate in the presence of various concentrations (0.01nM to 100nM) of everolimus or in normal K1 medium from day 1 for indicated experiments. The number of viable cells was determined by MTS assay using the CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega,

Madison, WI, USA) according to manufacturer's instructions. The absorbance was read at 492 nm with a microplate reader (Labsystems Multiskan RC., Haverhill, MA, USA).

BrdU uptake proliferation assay

Alternatively, cell proliferation was assessed using BrdU Cell Proliferation ELISA Kit (Cat. No. 1 647 229, Roche Applied Science, Basel, Switzerland), which quantifies cell proliferation by measuring DNA synthesis. Assay was performed according to manufacturer's instructions and results were expressed as mean absorbance of the samples in an ELISA plate reader (Molecular Devices Uvmax Reader) at 450 nm with a reference wavelength of 690 nm.

Apoptosis Assays

We determined apoptosis by 3 different methods. First, western blots (detail described in *Western blot analysis*) were performed to analyze the expression of caspase-3, a well characterized apoptosis marker.

Second, apoptosis was quantified by analyzing FACS for annexin V. Cell cultures of Cy/+ TECs were harvested by trypnization and double-stained with propidium iodide (PI) and fluorescein isothiocyanate-conjugated annexin V (Sigma, St. Louis) at day 3. Flow cytometry was performed with FACScan (Becton Dickinson) and the results were analyzed with the FACScan cellquest software Cells PI^-/Annexin V^+ were defined as early apoptotic cells, PI^+/Annexin V^+ as late apoptotic, and PI^+/Annexin V^- as dead cells. H_2O_2 treated TECs served as positive controls.

Third, the APOPercentage Apoptosis Assay (Biocolor, Belfast, Northern Ireland) was used to quantify apoptosis.[106] This assay discriminate apoptotic cells from necrotic cells as apoptotic cells undergo the membrane "flip-flop" event when phosphatidylserine is translocated to the outer leaflet. Cultures of Cy/+ were gently washed twice with PBS and medium were replaced by 100 µL apopercentage dye and incubatedat 37°C at day 3. After 1h, the cells were again washed twice with 100 µL PBS to remove un-trapped dye and 100 µl/well apopercentage dye release reagent was added for 10 min. The absorbance of the cell bound dye recovered in solution was measured using a microtitre plate colorimeter reader at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). TECs incubated with 1mM H_2O_2 for 1h were served as positive controls.

Western blot analysis

Western blot was done by standard methods. Briefly, 10 to 20 µg proteins extracted from cultured cells were boiled for 5 minutes, resolved in non-reducing SDS/PAGE and then subjected to Western blot analysis. The following antibodies were used: rabbit anti-phospho-mTOR (Ser2448), rabbit anti-phospho-mTOR (Ser2481), rabbit anti-phospho-p70 S6 kinase (Thr389), rabbit anti-phospho-p70 S6 kinase (Thr421/Ser424), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (Cell signaling Technology, Beverly, MA, USA), rabbit anti-caspase-3 (Cell signaling Technology, Beverly, MA, USA), and rabbit anti-actin (Sigma, St. Louis, MO, USA).

Aggregates formation and quantification

At day 1, TECs from Cy/+ and +/+ rats were harvested and seeded at 1 x 10^5 cells/ml into 6-well plates pre-coated with 1% tissue culture grade agar. Various

concentrations of everolimus (from 1 nM up to 100 nM in DMSO) or equivalent amount of solvent were added into the cell culture at the moment of plating on agar. At day 3, cell aggregates were formed and immobilized by embedding into collagen (2 g/l collagen type I solution (Vitrogen 100), 20mM HEPES (pH 7.4), 25mM NaHCO3) at 37°C for 1 h. Cell aggregates were fixed for 30 min with 4% FA/PBS at 37°C. Fluorescein-phalloidin (10 µg/ml in PBS) was applied for 1 hour at RT to stain F-actin outlining individual cells. Hoechst (10 µg/ml in PBS) was used to stain nuclei for 5 min at RT. Gels were mounted in mowiol/DABCO. Confocal images of aggregates were taken using Leica SP2 microscope equipped with an oil-immersion objective (40x, 1.3 NA, Leica Microsystems). Aggregate size was quantified using LSM Image Browser software (Zeiss).

Statistical analyses

Cell population doubling time was calculated by plotting cellular growth or proliferation against time. The everolimus concentration required to inhibit cell growth by 50% (IC_{50}) was calculated by interpolation of dose-response curves using GraphPad Prism 4 software. Statistical analyses were performed by one-way ANOVA with the Newman-Keul's post-hoc test. All data are expressed as means ± SEM and $P<0.05$ was considered as statistically significant.

Results

Tubular epithelial cells form Cy/+, compared to +/+, have a lower cell population doubling time in monolayer cultures

To investigate the proliferation capacity of +/+ and Cy/+ TECs, we determined the time of cell population doubling in monolayer cultures in the presence of absence of growth hormones. Total protein measurement and a colorimetric method gave similar results. Figure 1 shows that, with cell number assessed by colorimetric assay, the doubling time was significant lower of Cy/+ compared to +/+ in the absence of EGF (Cy/+ 15.5±0.6h *vs.* +/+ 21.9±4.1h, $p < 0.05$). Addition of EGF to the cell medium from day 1 to day 3 decreased the doubling time in both cell types, however Cy/+ still significantly proliferated faster than +/+, suggesting a preserved proliferation capacity despite basal high proliferation rate in Cy/+ TECs (data not shown).

Everolimus inhibited in dose-dependent manner and increase in Cy/+ tubular epithelial cell number by inhibition of proliferation

To explore the effects of everolimus on Cy/+ TECs, we assessed the cell number by colorimetric assay in the presence of varying concentration of everolimus (0.01 nM to 100 nM). Figure 2A shows that, exposure of Cy/+ TECs to everolimus for 2 days resulted in a dose-dependent decrease of cell number (IC_{50} 0.07±0.08 nM), starting from a concentration as low as 0.1 nM (0.1 nM vs 0 nM, $p < 0.05$; 0.01nM vs 0nM > 0.05) with no additional effect at higher everolimus concentration (0.1 nM vs 100 nM, $p > 0.05$) To assess whether the effect of everolimus on cell number that we had observed by the colorimetric assay was due to inhibition of proliferation, we used a BrdU uptake assay. Cells were cultured in the presence of varying doses of everolimus and BrdU was added at day 3. As shown in Figure 2B the effect on

proliferation mirrored that which we had seen on the colorimetric cell number assay. Everolimus showed a clear dose-dependent effect on DNA synthesis of Cy/+ tubular epithelial cells, starting to reduce cell proliferation at dose of 0.1 nM everolimus (0.1 nM vs 0 nM, $p < 0.05$; 0.01nM vs 0nM > 0.05; 0.1 nM vs 100 nM, $p > 0.05$). The proliferation inhibitory concentration of everolimus determined by BrdU uptake (IC_{50} 0.07±0.08) was similar to the IC_{50} assessed by colorimetric assay, indicating that the effect of everolimus on cell number was due to the direct inhibition on cell proliferation.

Everolimus did not reduce cell number by apoptosis

A possible explanation for the anti-proliferative effect of everolimus on tubular epithelial cells was that everolimus caused an increase in apoptosis, as this had been described for other cell types . Caspase 3, the convergence point of intrinsic and extrinsic apoptotic pathways, is a well-characterized readout for the presence of apoptosis. We therefore determined the expressions of caspase-3 by Western Blot and compared to untreated Cy/+ tubular epithelial cells, everolimus up to a concentration of 100 nM did not increase the expressions of proform (32KD) and activative form (17 kD) of caspase-3 (Figure 3A). Apoptosis is characterized by morphologic changes resulting in the exposure of phasphatidylserine on the outer membrane surface, which can be recognized by anti-Annexin V antibody. FACS analysis with anti-Annexin V antibody and no-vital dye propidium iodide (PI) discriminate viable ($Annexin^-PI^-$), early apoptotic ($Annexin^+PI^-$) and late apoptotic or necrotic cells ($Annexin^+PI^+$). Figure 3B shows that everolimus did not increase early or late apoptotic/necrotic cells in any concentrations. The number of $Annexin^+PI^+$ and $Annexin^+PI^-$ cells in control and everolimus treated samples could be due to the

damage incurred during trypsinization procedure, which could mask a possible effect of everolimus on cell apoptosis. Thererfore, we measured apoptosis using the apopercentage assay, which detects membrane alterations (phosphatidylserine flip) without prior trypsinization. Figure 3C shows that only few cells are stained positive for apoptotic cells in the presence of everolimus. Quantification of apoptotic cells by the apopercentage assay confirms that everolimus up to a concentration of 100 nM did not increase the number of apoptotic Cy/+ tubular epithelial cells (Figure 3D). Taken together, these results of 3 independent methods to detect apoptosis showed that the effect of everolimus on cell number was not due to increased apoptosis.

Everolimus dose-dependently decreased phosphorylation of mTOR and its downstream effectors S6 kinase and S6

To elucidate molecular mechanisms of everolimus induced inhibition of cell proliferation we determined the expression of mTOR pathway targets by western blots Figure 4A shows that two phosphorylation sites of mTOR were dose-dependently reduced by everolimus in Cy/+ tubular epithelial cells. Reduced expression of p2481 mTOR was evident at an everolimus concentration of 0.1 nM. A higher everolimus concentration further reduced p2481 mTOR expression reaching a nadir at 1 nM. A reduction of p2448 mTOR expression was seen at an everolimus concentration of 1nM and showed no further inhibition with higher concentration. Figure 4B shows everolimus inhibited the phosphorylation of S6 kinase, a direct downstream target of mTOR. The expression of p389 S6K and p421/424 S6K were dose-dependently reduced by everolimus, starting from a concentration as low than 0.1 nM. Dose-dependent decreased S6K activity was further confirmed by western blot analysis of p235/236 S6, a direct substrate of S6 kinase. These results show a

dose-dependant effect of everolimus on mTOR, S6 kinase and S6 phosphorylation in Cy/+ tubular epithelial cells and demonstrate these effects at same dose range that inhibited proliferation.

Everolimus reverted the increased diameter of Cy/+ tubular epithelial cell aggregate to the size of +/+

To study whether everolimus affects cyst formation *in vitro*, we cultured primary Cy/+ and +/+ tubular epithelial cells in suspension system with the aim to generate a 3D cell culture model for cystogenesis. Cy/+ and +/+ tubular epithelial cells formed aggregates 48 hours after plating. Figure 5A and C showed that the diameter of tubular epithelial cell aggregates originated from Cy/+ compared to +/+ was significantly higher (50±2 μm *vs*. 33±1 μm $p < 0.0001$). However the increase in aggregate size could not be attributed to appearance of luminal space inside the aggregates as confocal imaging demonstrated that there was no lumen in the absolute majority of either Cy/+ or +/+ aggregates (Figure 5B). The concentration of Cy/+ aggregates was lower than of +/+ (159±30 vs. 260±35 aggregates per 1 mm3, respectively) suggesting that some of the larger aggregates might have been formed by aggregate fusion. Both 1nM and 100 nM Everolimus significantly reduced the diameter of Cy/+ aggregates from 50±2 μm to 34±1 μm and 36±2 μm ($p < 0.0001$), respectively, to the range of +/+ aggregates. The diameter of +/+ aggregates remained unchanged by everolimus at a dose range of 1-100 nM ($p = 0.298$ and $p = 0.232$; Figure 5C and D). These results suggest that everolimus selectively inhibits either formation or growth or both of these parameters of Cy/+ tubular epithelial cell aggregates.

Discussions

Our study demonstrated that 0.1 nM was the lowest concentration of everolimus that affected cell number and DNA synthesis with no effect on apoptosis. The inhibition of cell proliferation was tightly linked to the reduced phosphorylation of mTOR, S6K and S6. In a 3D culture system, everolimus reduced the size of Cy/+ aggregates, the first step of cyst/lumen formation.

In our *in vitro* culture system, the doubling time of Cy/+ TECs was significantly lower than +/+ TECs, which was in line with the in vivo finding that the proliferation index in the kidney was higher in Cy/+ than +/+ [63, 81]. The everolimus dose inhibiting S6K activity was remarkable low (0.1 nM) in Cy/+ tubular epithelial cells. The sensitivity of cells to everolimus or rapamycin shows large variation in different cell types or species. 0.1 ng/ml (approximately equal to 0.1 nM) rapamycin in mouse mesangial cells [107], 0.5 nM and 20 nM rapamycin in human breast cancer cell MCF-7 and MDA-MB-231, respectively, decreased S6K activity [108]. Interestingly, in our study S6K activity was tightly linked to cell proliferation. A similar finding was reported by Lock et al. in mouse mesangial cells, suggesting a dominant role of the mTOR/S6K pathway in both cell types [107]. The level of rapamycin (0.5 nM for MCF-7 and 20 nM for MDA-MB-231), which inhibited S6K phosphorylation in MCF-7 and MDA-MB-231 cancer cells did not inhibit cell proliferation [92, 108]. The reason for this apparent difference in the anti-proliferative effects is due to the activation of multiple mitogenic pathways such as p27 and/or c-Myc, which confer resistance to rapamycin [1, 109, 110].

Currently blood level of everolimus or rapamycin achieved in most animal studies and clinical trials is between 4.5-14 nM (ng/ml) [60, 92, 107]. In our previous *in vivo* study,

we achieved blood trough levels of 5-7 nM by daily administration of everolimus (3mg/kg) using gavage [63]. In the current study a 0.1 nM everolimus level efficiently inhibited cell proliferation *in vitro*, which was in line with our previous *in vivo* study in which 0.5 nM blood rapamycin levels retarded cyst growth in Cy/+ rats [62]. Similarly, low everolimus dose (0.3 mg/kg) exerted a significant anti-proliferative effect on mesangial cells in a rat model of glomerulonephritis, which confirms the previous finding by Lock et al that 0.1 nM rapamycin *in vitro* inhibited renal mesangial cell proliferation [111].

Apoptosis is increased in polycystic kidneys and occurs in a similar rate in both cystic and non-cystic tubules [2, 73]. In a previous study in Cy/+ rats, TUNEL staining showed apoptosis in most cells of a particular tubule section whereas other tubules showed no apoptosis, suggesting that a focal micro-environment is required to initiate apoptosis [73]. In our study cultured Cy/+ cells had a low apoptotic rate, suggesting that Cy/+ cells were not intrinsically pro-apoptotic and further supports the hypothesis that in polycystic kidneys external pro-apoptotic signals are required to induce apoptosis.

In the present study, we assessed apoptosis by several methods: western blot of caspase 3, the converge point of intrinsic mitochondrial pathway and extrinsic death receptor-mediated pathway; annexin V/PI FACS analysis, which detects both early and late apoptotic cells; the APOPercentage Apoptosis Assay, which only measures early apoptotic cells. All three methods showed that everolimus had no effect on apoptosis induction in Cy/+ tubular epithelial cells. It has been shown that mTOR inhibitors within the clinical concentration did not induce apoptosis in several cancer cells and non-cancer cells, suggesting the function of mTOR inhibitors rather as

cytostatic effectors by arresting cells at G1 phase than as apoptosis inducers [107, 108, 112]. Indeed in our animal models, everolimus or rapamycin treatment did not induce the shrinking of cystic kidney but only stopped its growth [62, 80].

To elucidate the mechanisms of ADPKD cyst formation in vitro, a 3D culture of primary renal epithelial cells was established with primary cells isolated from +/+ and Cy/+ Han:SPRD rats. Classic *in vitro* cystogenesis involves in three major steps: 1) aggregates formation, 2) hollow lumen formation by vesicle exocytosis and luminal cell apoptosis, 3) cyst expansion [113]. In our study primary Cy/+ and +/+ TECs were collected at day 1 and were cultured in suspension in plates pre-coated with 1% agar to prevent adhesion, leading to the formation of cell aggregates in 48 h. However, the majority of aggregates contained no lumen; rarely aggregates with small lumen were observed. The efficiency of luminal aggregate formation in our primary TECs was lower than the dog renal epithelial cell line MDCK cells, probably due to the high resistance of primary cells to cell apoptosis and lower capacity to form polarized cell sheets in the absence of other cell types relative to MDCK cells. Inhibition of apoptosis prevented cyst formation in the 3D cultured MDCK cells [114] and the low rate of apoptosis found in Cy/+ may explain the absence of lumen in our model. In a previous study by Pey et al. cystic structures that were formed few hours after plating primary cells from Cy/+ Han:SPRD rats, markers were not showed to prove luminal formation [115].

We showed that Cy/+ cells formed significantly larger aggregates than +/+ cells. Cy/+ cells demonstrated a wider range of aggregate sizes than +/+ cells but with a similar lower limit, which suggested there was a diversity in proliferation rates of Cy/+ cells

spanning from normal to higher than normal values. Treatment of Cy/+ cells with 1-100 nM everolimus significantly reduced aggregate diameter, which was tightly associated with the dose-dependant reduction of cell proliferation rate and mTOR activity by everolimus in monolayer Cy/+ cell culture. Interestingly, the lower limit of Cy/+ aggregate size stayed unchanged suggesting that everolimus treatment selectively affected Cy/+ cells with higher than normal proliferation rates. Thus, inhibitors of mTOR might reduce renal cyst formation and growth by suppressing mTOR-mediated cell proliferation.

In conclusion, 0.1 nM could be the lowest biological dose for everolimus to inhibit Cy/+ TEC growth. A low level of everolimus selectively reduced Cy/+ aggregate size, which is paralleled with the inhibition of mTOR pathway, but not with the induction of apoptosis.

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Legends

Figure 1.

Viable +/+ (open circles) and Cy/+ (closed circles) TECs were assessed by colorimetric MTS assay from 0 h (day 1) to 48 h (day 3). All experiments were performed in triplicates, bar indicate mean (SEM). Results were reported as OD value.

Figure 2.

Cy/+ TECs were treated with various concentrations of everolimus (0.01 to 100 nM) for two days. At day3 (A) MTS and (B) BrdU assay were used to determine viable cell number and DNA synthesis of Cy/+ TECs, respectively. All experiments were performed in triplicates, bar indicate mean (SEM). Results were reported as OD value. ($p<0.0001$ versus everolimus 0 nM, double asterisk)

Figure 3.

Cy/+ TECs were treated with various concentrations of everolimus (0.01 to 100 nM) for 1 day for apoptosis analysis at day 3. (A) Whole cell lysate was subjected to Western analysis to exam the expression of full-length (32 kD) and cleaved (17 kD) caspase-3. Western blot of actin serves as internal control. Representative blot is shown. (B) Representative FACS analysis of annexin V and PI staining of Cy/+ TECs. (C) Cy/+ TECs were stained by APOPercentage kit. Apoptotic cells are stained red. (D) Quantification of results from APOPercentage staining as OD value. 1 mM H_2O_2 treated TECs served as positive control. Representative data are shown. Error bar indicated SEM of triplicate culture.

Figure 4.

Cy/+ TECs were treated with various concentrations of everolimus (0.01 to 100 nM) for 1 day. Cell lysates were collected at day 3. (A) Western analysis of total, p2481 and p2448 mTOR are shown. (B) Western analysis of total, p389, p421/424 S6 kinase and p235/236 S6 are shown. Western blot of actin serves as internal control. Representative blot is shown.

Figure 5.

(A) Representative images of +/+ and Cy/+ TEC aggregates grown in collagen type I (0.15 g/L) gel. (B) Double staining for cells' outline (F-actin; green) and nuclei (blue) indicate absence of lumen in Het aggregates. Bar 5 μm. (C) The diameter of Cy/+ and +/+ TEC aggregates after 48 hours of growth on non-adhesive plates in the presence of 1 nM or 100 nM everolimus or solvent (DMSO). Everolimus significantly ($p<0.0001$, double asterisk) reduced aggregate size of Cy/+ but not +/+ TECs. (D) Representative phase-contrast images of Cy/+ and +/+ TEC suspension cultures described in (C). Bar 30 μm.

Figure 1

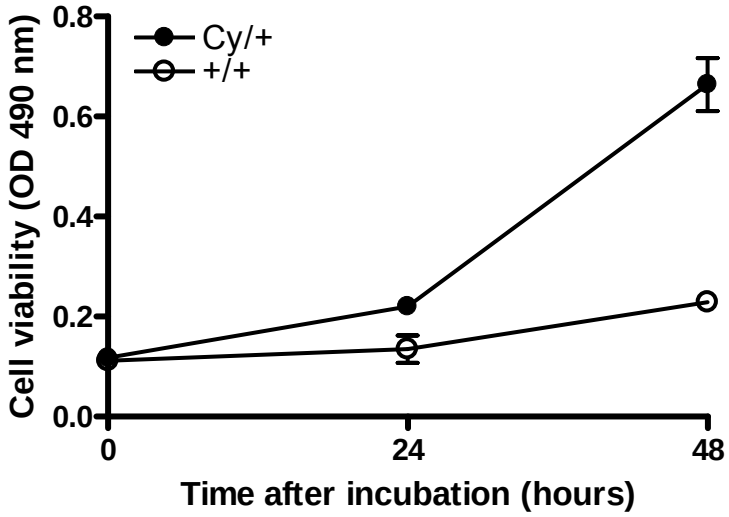


Figure 2

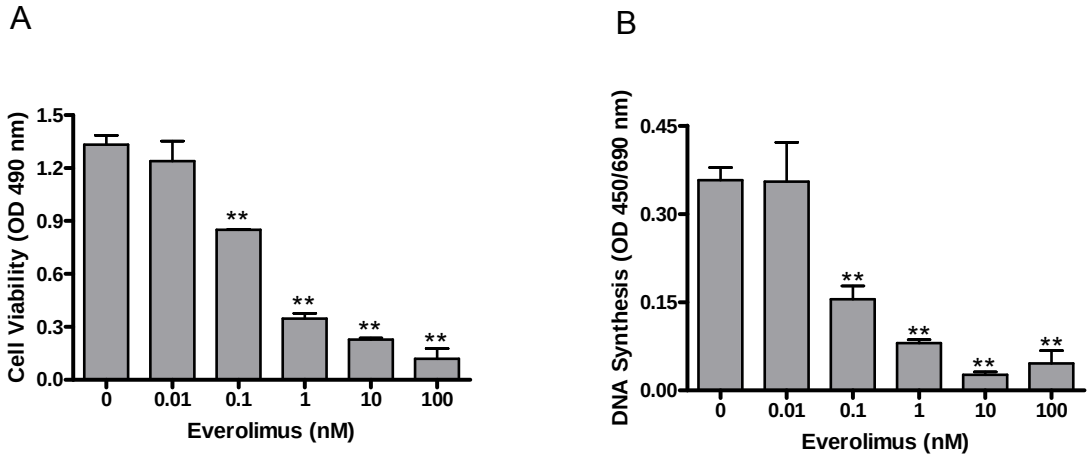
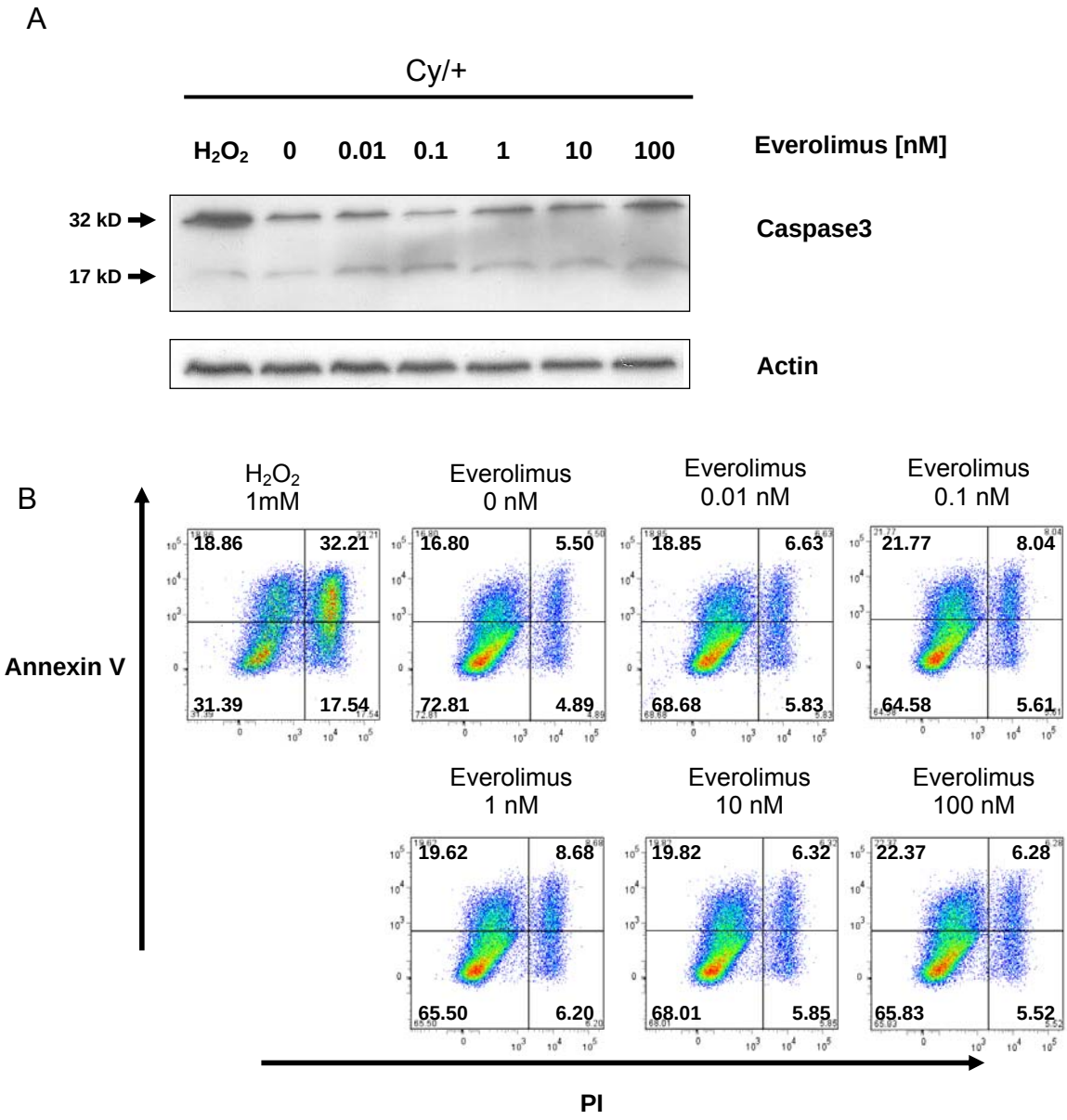
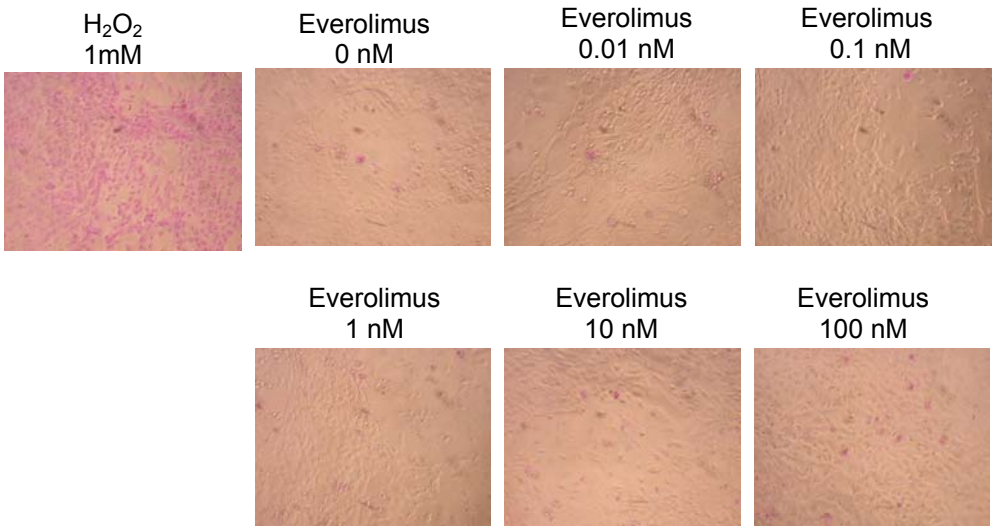


Figure 3


A
Cy/+
H2O2
0
0.01
0.1
1
10
100
Everolimus [nM]
32 kD
17 kD
Caspase3
Actin
B
H2O2 1mM
Everolimus 0 nM
Everolimus 0.01 nM
Everolimus 0.1 nM
Everolimus 1 nM
Everolimus 10 nM
Everolimus 100 nM
18.86
32.21
31.39
17.54
16.80
5.50
72.81
4.89
18.85
6.63
68.68
5.83
21.77
8.04
64.58
5.61
19.62
8.68
65.50
6.20
19.82
6.32
68.01
5.85
22.37
6.28
65.83
5.52
Annexin V
PI

C


H_2O_2 1mM
Everolimus 0 nM
Everolimus 0.01 nM
Everolimus 0.1 nM
Everolimus 1 nM
Everolimus 10 nM
Everolimus 100 nM

D

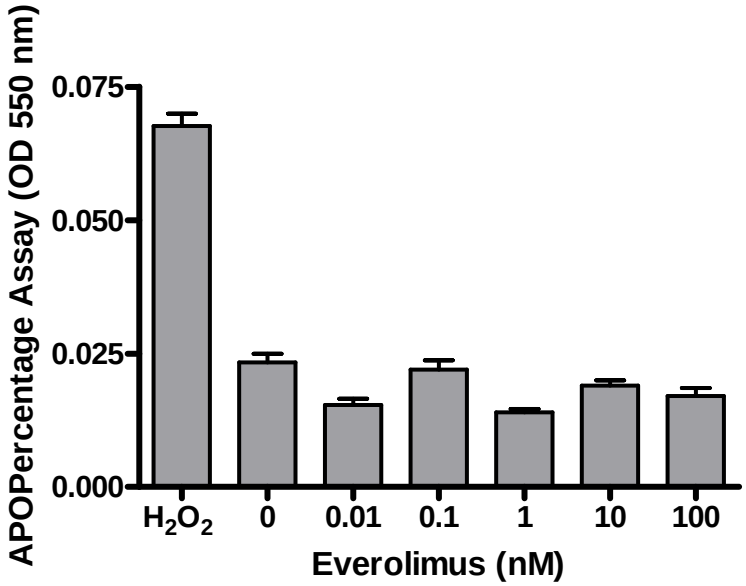
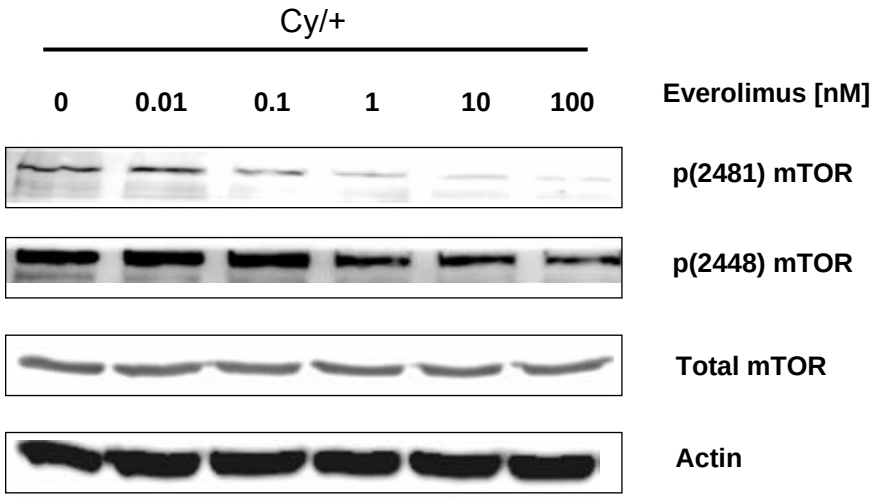

APOPercentage Assay (OD 550 nm)
0.075
0.050
0.025
0.000
H_2O_2
0
0.01
0.1
1
10
100
Everolimus (nM)

Figure 4

A


Cy/+
0
0.01
0.1
1
10
100
Everolimus [nM]
p(2481) mTOR
p(2448) mTOR
Total mTOR
Actin

B

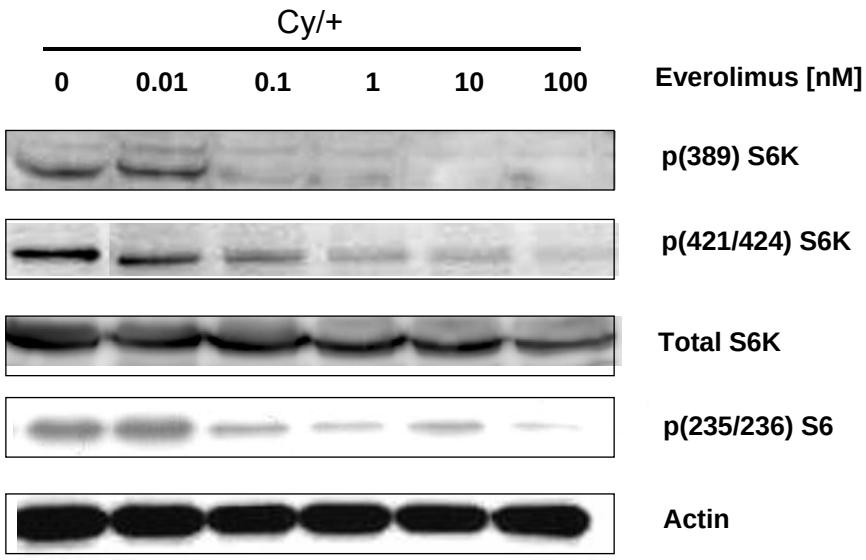
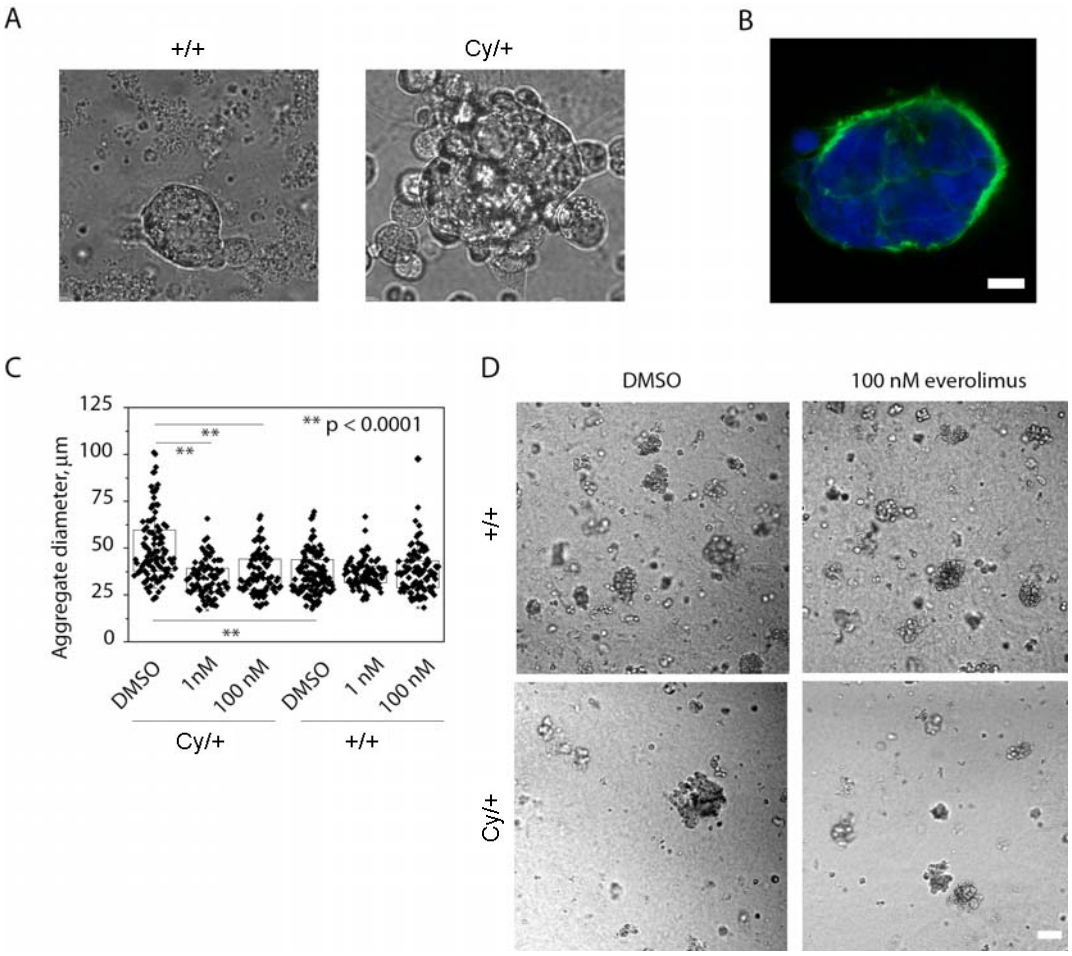

Cy/+
0
0.01
0.1
1
10
100
Everolimus [nM]
p(389) S6K
p(421/424) S6K
Total S6K
p(235/236) S6
Actin

Figure 5


A
+/+
Cy/+
B
C
Aggregate diameter, µm
125
100
75
50
25
0
** p < 0.0001
**
**
**
DMSO
1nM
100 nM
DMSO
1 nM
100 nM
Cy/+
+/+
D
DMSO
100 nM everolimus
+/+
Cy/+

Chapter 5

Conclusion and perspectives

Conclusion and discussion of current work

Because of the important role of mTOR signaling in cell proliferation and growth, mTOR inhibitors such as sirolimus and everolimus have been used to treat cancer patients. Our findings demonstrate that everolimus retarded cyst growth and preserved renal function, which was associated with decreased cell proliferation in renal tubules in Cy/+ Han:SPRD rats. Together with previous study of sirolimus, we showed a class effect of mTOR inhibitors on disease progression in polycystic kidney disease.

The effect of mTOR inhibitor withdrawal has not been studied so far. In the second study, we showed that everolimus pulse treatment (everolimus withdrawal) and everolimus continuous treatment had same anti-cystic and reno protective effects, although a partial reactivation of S6 kinase and recovery of cell proliferation were observed 7 weeks after cessation of everolimus treatment.

Recent studies show that the timing of cystogenesis is critical for disease progression in PKD [6, 18]. Inactivation of these genes in adult mice only causes a slower progressive cystic disease. Our current study showed that the retardation of cyst growth by mTOR inhibitor everolimus at an early age (4-9 weeks) in Cy/+ rats led to a non-cystic/less-cystic hypertrophic phenotype seven weeks after everolimus withdrawal. These findings therefore suggest that blocking cystogenesis in the early time could change the course of disease progression in PKD.

In our third study, we established an *in vitro* primary cell culture model for PKD by showing a hyper-proliferative renal tubular epithelial cells derived from Cy/+ rats. We demonstrated that 0.1 nM was the lowest biological dosage of everolimus to inhibit cell proliferation, which was tightly linked to mTOR/S6 kinase activity. We showed that the level of apoptosis was low in Cy/+ cells in vitro and everolimus had

no effect on apoptosis in Cy/+ cells. mTORC2 plays an important role in cell survival and provides resistance to apoptosis [92]. Indeed, we showed in the second study that the activity of mTORC2 was up-regulated in Cy/+ rats and resistant to continuous everolimus treatment. This is because that suppression of mTORC2 activity required a very high level of mTOR inhibitor (μM) [92]. An in vivo mouse study reported that rapamycin reversed cyst growth and enhanced apoptosis in cystic kidneys [45]. However the blood concentration of rapamycin in this study was not known. The sensitivity of mTORC2 depends on the intracellular phosphatidic acid (PA) level, which is often up-regulated in tumour cells and binds/stabilizes mTOR complex, thereby rendering the resistance of mTOR complex to everolimus/rapamycin. Therefore combination therapy of PA inhibitor and everolimus could be tested *in vitro* and in adult Cy/+ rats.

Model of cyst formation and expansion

After literature study of research papers and review articles published in the field of PKD in recent three years, we summarized the most important factors for cyst formation, expansion and maintainence of cystic phenotype (Table 1). We can see the importance of disrupted PCP, defect cell migration, and high proliferation/ apoptosis background as driving forces to initiate cyst formation. The disrupted PCP and cell migration are caused by germline mutation or inflammation mediated reduction of PKD protein dosage in primary cilia or cell adhesion junctions. Once a cystic phenotype is generated, the focal cyst may produce mitogenic and inflammatory factors to expand itself and transform its surrounding tubule into a cystic phenotype.

Table 1 Important factors for cyst formation and expansion

	Factors	Causes
Cyst formation	Reduced PKD protein dosage	• Germline mutation • Defect of intracellular PKD protein trafficking caused by inflammatory factors like TNF-a
	Disrupted PCP and cell migration	• Reduced PKD protein dosage leading to impaired cilia • Impaired cell-cell adhension and cell-matrix adhension
	High proliferation and apoptosis background	• In kidney developmental period • Local infection • Local injury
Cystic cell phenotype	• Disrupted epithelial cell polarity • Secretary phenotype • Proliferative phenotype • Activated inflammatory factors (NF-kb and TNF-a)	

	Factors	Causes
Cyst expansion	High secretion	• Loss of cell polarity • Defect of calcium/cAMP signals

High proliferation	• Loss of cell polarity • Defect of calcium/cAMP signals
Enhanced inflammation	• Active NFkb in cystic epithelial cells induced local inflammation
Enhanced apoptosis	• Local inflammation induced noncystic cell apoptosis

Perspective and outlook

Timing of treatment and inhibition of PCP pathway in ADPKD

The focus of PKD research is shifting from primary cilium to the timing of cystogenesis and PCP in PKD in recent years. With our rat PKD model and the tool of everolimus, we could study the effect of treatment time on ADPKD based on the timing of cystogenesis and cyst development. Further studies with everolimus may involve three treatment time points (postnatal period, developing period, adult period), which would be of great relevance to clinic situation.

Seven years ago, before discovery of PCP issue in PKD, the effects of PPAR-gamma agonist has been tested in *Pkd1* knockout mice [116]. Maternally administered pioglitazone, a PPAR-gamma agonist, improved embryonic survival of mutant mice and reduced the extent of renal cystogenesis, however the mechanism is unknown. It has been shown that PPARγ agonists repress the Wnt/β-catenin signaling [117]. Therefore we hypothesize that PPARγ agonists inhibit cystogensis via suppressing Wnt/β-catenin signals and thereby rescue the impaired PCP in PKD. With the collaboration with our Chinese partners, who developed several new PPARγ agonists, we are able to test the effect of PPARγ agonists in our rat model of ADPKD.

Issue of inflammation in ADPKD

Very recently, the role of immune system/inflammation as a non-genetic factor (third hit) has been emphasized by several top scientists in this field of PKD. With our everolimus withdrawal model, we can study whether inflammatory/environmental factors such as TNF-α can accelerate disease progression after everolimus withdrawal. In addition, in a very recent study, nuclear factor-kB (NF-kB) has been found to be up-regulated in *pkd1* deficient cells and cystic cells [31]. NFkB is a transcription factor thought to play an important role in the onset of inflammation. It would be interesting to see whether inhibition of NFkB retards disease progression in Cy/+ rats by preventing the recruitment of inflammatory cells to cystic tissues. Sirolimus and everolimus were initially used as immunosuppressants and can inhibit the activity of macrophages, dendritic cells and conventional T cells. A recent study has also shown that mTOR deficiency leads to induction of regulatory T cells [118]. Therefore, we can not exclude that the inhibition of disease progression by everolimus in Cy/+ rats is partly mediated by inhibition of immune system or induction of regulatory T cells.

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